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Publication date:
2017

Document version
Peer reviewed version

Citation for pulished version (APA):
Dammann Andersen, A. (2017). Cerebrospinal Fluid Biomarkers For Parkinson's Disease and L-DOPA-induced Dyskinesia. Syddansk Universitet. Det Sundhedsvidenskabelige Fakultet.

Cerebrospinal fluid biomarkers for Parkinson's disease and L-DOPA-induced dyskinesia

PhD-thesis

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July 2017

Front page illustration made by Frederik Dammann Andersen

Printed by Print & Sign, SDU.

Cerebrospinal fluid biomarkers for Parkinson's disease and L-DOPA-induced dyskinesia

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated with love to Ture, Bodil, Frederik, Asbjørn, Line, Hannibal and Jonathan

List of publications and manuscripts:

I. **Cerebrospinal fluid biomarkers for Parkinson's disease – a systematic review,**
ANDREAS DAMMANN ANDERSEN, Binzer M, Stenager E, Gramsbergen JB.
Acta Neurol Scand. 2017 Jan; 135(1):34-56

II. **Cerebrospinal fluid levels of catecholamines and its metabolites in Parkinson's disease: effect of L-DOPA treatment and changes in levodopa-induced dyskinesia.**
ANDREAS DAMMANN ANDERSEN, Blaabjerg M, Binzer M, Kamal A, Thagesen H, Kjaer TW, Stenager E, Gramsbergen JB
J Neurochem. 2017 May;141(4):614-625

III. **Changes in kynurenine pathway metabolism in Parkinson patients with L-DOPA-induced dyskinesia.**
JESPER HAVELUND, ANDREAS DAMMANN ANDERSEN, Binzer M, Blaabjerg M, Heegaard NHH, Stenager E, Faergeman NJ, Gramsbergen JB

IV. **L-Tryptophanol for the quantification of prefibrillary proteins in CSF and plasma in Parkinson's disease**
ANDREAS DAMMANN ANDERSEN, Jones G, Blaabjerg M, Binzer M, Gramsbergen JB, Platt B, Stenager E
(*Manuscript*)

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LIST OF ABBREVIATIONS:

3-HK: 3-hydroxykynurenine
3-HANA: 3-hydroxyanthranilic acid
3-OMD: 3-O-methyldopa
5-HIAA: 5-hydroxyindoleacetic acid
5-HT: 5-hydroxytryptamine (serotonine)
AADC: Amino acid decarboxylase
Aβ: Amyloid beta
ab: antibody
AD: Alzheimer's disease
ANA: Anthranilic acid
AUC: Area under the curve
α-syn: α-synuclein
BBB: Blood brain barrier
BDNF: Brain derived neurotrophic factor
cAMP: cyclic adenosine monophosphate
CBD: Corticobasal degeneration
CM: Centromedian nucleus of thalamus
CSF: Cerebrospinal fluid
DA: Dopamine
DAT-SPECT: Dopamine transporter-single photon emission tomography
DOPAC: 3,4-dihydroxyphenylacetic acid
DOPAL: 3,4-dihydroxyphenylacetaldehyde
DMV: Dorsal motor nucleus of vagus
DLB: Lewy Body dementia
D1-receptor: Dopamine receptor sub-stype
ELISA: Enzyme linked immunosorbent assay
ENS: Enteric nervous system
ERK: Extracellular signal-regulated kinase
GCase: Glucocerebrosidase
GPi: Internal globus pallidus
GPe: External globus pallidus
GBA1: Glucocerebrosidase gene
GD: Gaucher's disease
HVA: Homovanillic acid
HPLC: High performance liquid-chromatography
KYN: Kynurenine
KYNA: Kynurenic acid
KAT: Kynurenine amino transferase
KMO: Kynurenine 3-monooxygenase
LB: Lewy body
LC-MS: Liquid chromatography-mass spectrometry
L-DOPA: Levodopa
LED: Levodopa equivalent dose
LID: L-DOPA-induced dyskinesia
LRRK: Leucine rich repeat-kinase
LP: Lumbar puncture
MAO: Monoamine oxidase
MHPG: 3-methoxy-4-hydroxyphenylglycol
MIBG: metaiodobenzylguanidine
MMSE: Mini mental-state examination
MSA: Multiple system atrophy
MSN: Medium spiny neurons
MOCA: Montreal cognitive assessment
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MUGIc: 4-Methylumbelliferyl beta-D-glucopyranoside
NMDA: N-methyl-D-aspartate
NA: Noradrenaline
PD: Parkinson's disease
PD-N: Parkinson's disease, not treated with L-DOPA
PD-L: Parkinson's disease, L-DOPA-treated, non-dyskinetic
PD-LID: Parkinson's disease, L-DOPA treated, dyskinetic
PINK1: phosphatase and tensin homolog-induced putative kinase-1
PNS: Peripheral nervous system
PSP: Progressive supranuclear palsy
PF: Parafascicular nucleus of thalamus
QUIN: Quinolinic acid
RSBD: Rapid eye movement sleep-behavior disorder
ROS: Reactive oxygen species
ROC: Receiver operating characteristics
SNpc: Substantia nigra, pars compacta
STN: Subthalamic nucleus
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Trol: L-Tryptophanol
TRP: Tryptohpane
UDysRS: Unified dyskinesia rating scale
UPDRS: Unified Parkinson's disease rating scale
UKBBC: United Kingdom Brain Bank Criteria
VEGF: Vascular endothelial growth factor
VL: Ventral lateral nucleus of thalamus
XAN: Xanthurenic acid

Chapter 1: Resumé

1.1: English resumé

Parkinson's disease (PD) is the second most common neurodegenerative disorder next to Alzheimer's disease. It is characterized by a gradual onset of motor symptoms such as slowness of movement, resting tremor, rigidity and postural instability, related to the degeneration of dopaminergic neurons in substantia nigra, pars compacta (SNpc). Apart from this, patients also suffer from increasing autonomous dysfunction, e.g. drooling, urinary incontinence, constipation, and ortostatic hypotension. The risk of PD increases significantly with age, with approximately 1% of the population over 60 years suffering from PD. The disease process leading to PD may have been taking place for more than 10 year prior to the motor symptom debut, since motor symptoms only occur after more than 30% of the dopaminergic neurons in SNpc have deteriorated. The current treatment options do not include disease-modifying drugs, but only symptomatic treatment. The most effective symptomatic treatment for PD is the drug L-DOPA, which substitutes the loss of dopamine in the striatum. During the first years of treatment, this drug can have a marked effect on the patient's motor symptoms, but gradually the drug loses its effect. Apart from daily potentially severe fluctuations in treatment effect, patients begin developing side effects, such as potentially debilitating involuntary movements called L-DOPA induced dyskinesia (LID). In total there is a need for earlier diagnosis of PD, a better understanding of the LID pathogenesis and identifying patients at risk of developing LID

The aims of this thesis are: 1) To identify diagnostic biomarker candidates for PD in the cerebrospinal fluid (CSF) and blood plasma. 2) To identify biomarker candidates for LID in CSF and blood plasma.

In this study we included 26 patients with PD and 16 controls. Apart from CSF and blood sampling, PD patients were clinically rated using rating scales assessing the severity of PD (MDS-UPDRS part III), the severity of LID (UDysRS objective score), as well as a cognitive rating (MOCA, MMSE). 8 patients were not treated with L-DOPA, 18 patient were L-DOPA treated and 10 of them were dyskinetic. Samples were analysed using high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC-MS), western blotting, enzyme linked immune-assays (ELISA), Multiplex, and fluorescence assays.

Several potential diagnostic biomarker candidates were identified: PD patients had a significantly decreased concentration of the protein α-synuclein (α-syn) in CSF as well as an increased tetramer/monomer α-syn ratio. They had significantly decreased activity of the enzyme glucocerebrosidase in CSF. Using a potential prefibrillary amyloid marker, L-Tryptophanol (Trol), PD patients had significantly decreased Trol scores in CSF and significantly increased Trol scores in plasma. The CSF/plasma Trol ratio and the CSF Trol/CSF total protein-ratio increased differentiation between PD and controls. Several catecholamines were also decreased in L-DOPA-untreated PD patients.

In dyskinetic patients significant changes in tryptophan and catecholamine metabolic pathways were identified as well as an increased phosphorylation of the enzyme extracellular signal-regulated kinase (ERK 1/2).

The findings of this study may further our understanding of the underlying pathogenic pathways leading to PD and LID. Future longitudinal studies are needed to corroborate the usefulness of these biomarker candidates, including age matched healthy controls, more patients as well as patients with more severe LID.

1.2: Dansk resumé:

Parkinson's sygdom (PS) er den næsthyppigste neurodegenerative lidelse efter Alzheimer's sygdom. Sygdommen er karakteriseret ved en gradvis udvikling af bevægeforstyrrelser i form af langsomme bevægelser, hvilerysten, rigiditet og postural instabilitet. Dette er relateret til en degeneration af dopaminerge neuroner i substantia nigra, pars compacta (SNpc). Udover dette lider patienterne også af tiltagende autonome symptomer som f.eks. savlen, urininkontinens, forstoppelse og ortostatisk hypotension. Risikoen for PS stiger markant med alderen, og ca.1% af befolkningen over 60 år har PS. Udviklingen af sygdommen kan potentielt være begyndt mere end 10 år før udviklingen af bevægeforstyrrelser, da mere end 30% af de dopaminerge neuroner i SNpc er skadet før man udvikler bevægeforstyrrelser. Der findes ingen behandling, som kan bremse sygdommen, og den bedste symptomatiske behandling med stoffet L-DOPA, som erstatter den manglende dopamin i striatum, mister gradvist sin effekt efter flere års behandling. Udover at patienterne begynder at opleve daglige markante svingninger i effekten af L-DOPA, vil de også udvikle flere bivirkninger til behandlingen. Dette er blandt andet potentielt invaliderende ufrivillige bevægelser, kaldet L-DOPA-inducerede dyskinesier (LID). Samlet set er der et behov for tidligere diagnosticering af PS, en bedre forståelse af patogenesen bag LID samt at identificere patienter med risiko for at udvikle LID.

Målene med denne afhandling er: 1) At identificere diagnostiske biomarkør-kandidater i cerebrospinalvæsken (CSV) og blodplasma fra patienter med PS. 2) At identificere LID biomarkør-kandidater i CSF og blodplasma fra patienter med PS.

I dette projekt inkluderede vi 26 patienter med PS og 16 kontroller. Udover udtagning af CSV og blodprøvetagning blev PS-patienterne også klinisk vurderet. Dette blev gjort vha. en vurderingsskala for sværhedsgraden af bevægeforstyrrelse (MDS-UPDRS part III) samt sværhedsgraden af dyskinesi (UDysRS ojektiv score). Vurderingsskaler for kognitiv formåen blev også brugt til PS-patienterne (MOCA, MMSE). 8 PS-patienter modtog ikke behandling med L-DOPA, 18 patienter blev behandlet med L-DOPA, hvoraf 10 havde LID. Prøverne blev analyseret ved brug af high-performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC-MS), western blotting, enzyme linked immune-assays (ELISA), Multiplex og fluorescence-assays.

Flere mulige diagnostiske biomarkør-kandidater blev identificeret: Koncentrationen af proteinet α-synuclein (α-syn) var nedsat i CSV, og der var en øget α-syn tetramer/monomer-ratio. Aktiviteten i CSV af enzymet glucocerebrosidase var markant nedsat hos PS-patienter. Ved hjælp af den muligt præfibrillære amyloide markør, L-Tryptophanol (Trol), fandt vi en signifikant nedsat Trol-score i CSV og øget Trol-score i plasma hos PS-patienter. Både CSV Trol/plasma-Trol-ratio og CSV Trol/CSV total protein-ratio medførte en bedre skelnen mellem PS og kontroller. Koncentrationen af flere katekolaminer var også signifikant nedsat hos PS-patienter, som ikke fik L-DOPA.

Patienter med LID havde signifikante forandringer i tryptofan- og katekolamin-metabolismen og en øget fosforylering af enzymet extracellular signal-regulated kinase (ERK 1/2).

Disse fund kan bidrage til en bedre forståelse af de sygdomsmekanismer, som giver PS og LID. For at bekræfte brugbarheden af disse mulige biomarkører er der brug for longitudinelle studier inklusive alders-matchede raske kontroller, flere PS-patienter og PS-patienter med mere alvorlig LID.

Chapter 2: Introduction

2.1 A brief historical perspective:

200 years ago, in 1817, James Parkinson wrote "An essay on the shaking palsy"(Parkinson 2002). He discovered a pattern of symptoms that he believed encompassed a specific disease entity, the shaking palsy, which later came to bear his name. In the essay he described the insidious onset of this new disease; the unilateral mild weakness of an extremity and a mild resting tremor. And during progression, he noticed a bilateral spread of symptoms, a marked scarcity of movements, small handwriting and a more continuous and severe resting tremor ("agitation of the limbs"). Patients developed a gradually more pronounced stooped posture, shuffling gait, tendency of falling, inability to stop movements, drooling, sleep disturbance, constipation, incontinence, dysarthria, and dysphagia rendering the patient completely helpless in the latest stages of the disease. The inevitable fate of the patient is dismally described:

"The urin and faeces are passed involuntarily; and at the last, constant sleepiness, with slight delirium, and other marks of extreme exhaustion, announce the wished-for release."

James Parkinson believed the disease to be inflammatory of origin, potentially beginning in the superior part of medulla spinalis, slowly progressing to involve the medulla oblongata. Contrary to future findings, he did not believe the disease to involve cortical regions and did not identify the cognitive deficiency that affects these patients. Maybe the severe motor symptoms of the untreated patient precluded any evaluation of cognitive function.

2.2 About Parkinson's disease:

2.2.1 Cardinal symptoms

Whereas Parkinson saw resting tremor as one of the main clinical features of Parkinson's disease (PD), the current diagnostic criteria for PD allow for a more heterogeneous clinical

picture. In our systematic review of cerebrospinal fluid biomarkers(Andersen *et al.* 2016)(Appendix I), diagnostic criteria from several different authors have been used to correctly identify PD patients. Often cited, and also used in this project, is the United Kingdom Brain Bank criteria (UKBBC) to which several papers are cited(Gibb & Lees 1988, Hughes *et al.* 1992, Daniel & Lees 1993), albeit it seems Gibb et al were the first to produce the criteria(Gibb & Lees 1988). In the UKBBC, bradykinesia is the *main cardinal symptom* of the disease and either one of muscular rigidity, a 4-6 Hz resting tremor or postural instability is required as a supplementing symptom. *Exclusion criteria* includes repeated stroke and subsequent stepwise PD symptoms, repeated head injury, definite encephalitis, oculogyric crisis, neuroleptic treatment at onset, >1 affected relative, sustained remission, unilateral symptoms after 3 years, supranuclear gaze palsy, cerebellar signs, early severe autonomic signs, early severe dementia, Babinski's sign, cerebral tumour or communicating hydrocephalus, no L-DOPA-response, and neurotoxin exposure such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). For a definite diagnosis three supportive criteria have to be fulfilled: Unilateral onset, resting tremor, progressive disease, persistent asymmetry, excellent response (70-100%) to L-DOPA, severe L-DOPA-induced chorea, L-DOPA response >5 years, or a clinical course >10 years. One of the main differences between the UKBBC and other diagnostic criteria set forth (Koller 1992, Calne *et al.* 1992, Gelb *et al.* 1999), is that in the latter three bradykinesia is not viewed upon as the main cardinal feature in the clinically probable PD diagnosis. Calne et al(Calne et al. 1992) even allows for a clinically definite diagnosis of PD without the occurrence of bradykinesia, if either resting tremor or rigidity is asymmetrical. After commencing this PhD project, the Movement Disorder Society has produced a new set of more elaborate criteria for PD (Postuma *et al.* 2015). Again, bradykinesia (combined with either resting tremor or rigidity) is defined as the cardinal symptom of PD, combining slowness of movement (bradykinesia) and gradually declining amplitude during repetitive movements (hypokinesia/akinesia). New supportive criteria include clinically established olfactory deficiency or loss of cardiac sympathetic innervation indicated by the use of iodine-123-meta-iodobenzylguanidine (MIBG) scintigraphy. It is to be noted that abnormal functional neuroimaging (such as the much used dopamine transporter – single photon emission computer tomography (DAT-SPECT)) is not viewed upon as a supportive criterion for PD, whereas a normal functional neuroimaging excludes the PD diagnoses. Also red flag criteria are included that do not exclude a probable PD diagnosis but

has to be counterbalanced by supportive criteria. An important red flag is the absence of common premotor symptoms after 5 years of disease duration.

2.2.2. Demographics

A systematic review of incidence studies(Twelves *et al.* 2003) found that the methods used in the studies for identifying PD patients were heterogeneous and oftentimes insufficient. Thus, varying inclusion and exclusion criteria or unspecified criteria were used to diagnose PD patients in the base populations; sources of information also varied, and in some studies none of the patients had been examined as part of the study. Incidence definitions also varied. Some studies used time of diagnosis and others used time of symptom onset. Since there might be a certain delay between onset of symptoms and the first contact to a health care professional this might skew the incidence figures. The methodologically most similar studies(Granieri *et al.* 1991, Marttila & Rinne 1976, Kuopio *et al.* 1999, Mayeux *et al.* 1995, MacDonald *et al.* 2000) in the systematic review(Twelves et al. 2003) show a marked increase of yearly incidence in the +60 population, with the highest incidence often in the age between 70 and 79 years, and an average yearly incidence varying from 8-18/100.000/year. According to the Danish Movement Disorder Society (DANMODIS)(DANMODIS 2017), there are 5000-6000 patients with PD in Denmark. It has been suggested that men are more prone to develop PD than women(Kessler 1972). A large study including 17,205 elderly subjects from several European populations(de Rijk *et al.* 1997) did not find a marked gender difference, apart from more men being more affected after the age of 80 years. It has to be noted, though, that the diagnostic criteria used in that study were not as rigid, with the possibility of identifying subjects as having PD without showing bradykinesia. In the study the overall prevalence for elderly between 65-69 years was 0.9/100, increasing to 3.5/100 in the ages 85-89 years.

2.2.3 Medical treatment of Parkinson's disease

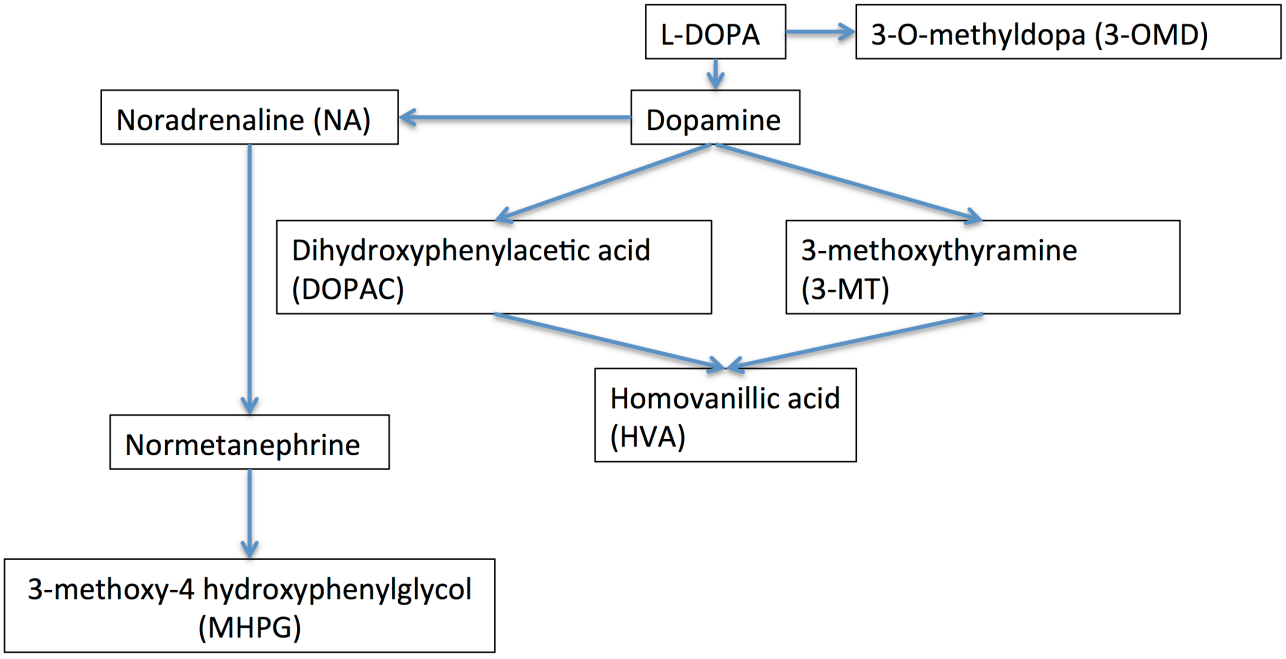

Figure 1: A depiction of the metabolic pathways for L-DOPA.

L-DOPA:

L-DOPA is the precursor to the neurotransmitter dopamine (DA). Unlike DA, L-DOPA has the ability to cross the blood brain barrier (BBB), being converted into DA in especially dopaminergic neurons by decarboxylation (although other cell types also take up L-DOPA)(see figure 1). Almost 50 years after the initial study using L-DOPA treatment on PD patients(Cotzias 1968), it continues to be the most efficient symptomatic treatment available for PD. One year after their initial discovery, the same author published results from treating PD patients with gradually increasing L-DOPA doses(Cotzias *et al.* 1969). This provided a sustained response in PD patients in terms of decreased akinesia, rigidity and tremor as well as attenuation of autonomic dysfunction. In several cases patients even returned to work. They also showed psychological improvements in terms of "amelioration of outlook, interest and behavior", but in some cases also a marked mental improvement described as an "awakening"(Cotzias et al. 1969). The treatment responses were not homogenous and side effects occurred at a high rate. Many patients suffered from nausea, and it was quickly hypothesized that a stimulation of area postrema, which is not protected by the BBB, was due to the peripheral L-DOPA-decarboxylation (approximately 50-80% intestinal decarboxylation)(Marsden & Donaldson 2012). In the seventies L-DOPA drugs combined with decarboxylase inhibitors benserazide (Madopar®) and carbidopa (Sinemet®, which literally

is latin for "without vomiting") were introduced to avoid this issue(Marsden & Donaldson 2012). The decarboxylase inhibitors were used due to their inability to cross the BBB, and this combination dramatically increased the amount of L-DOPA available in the CNS.

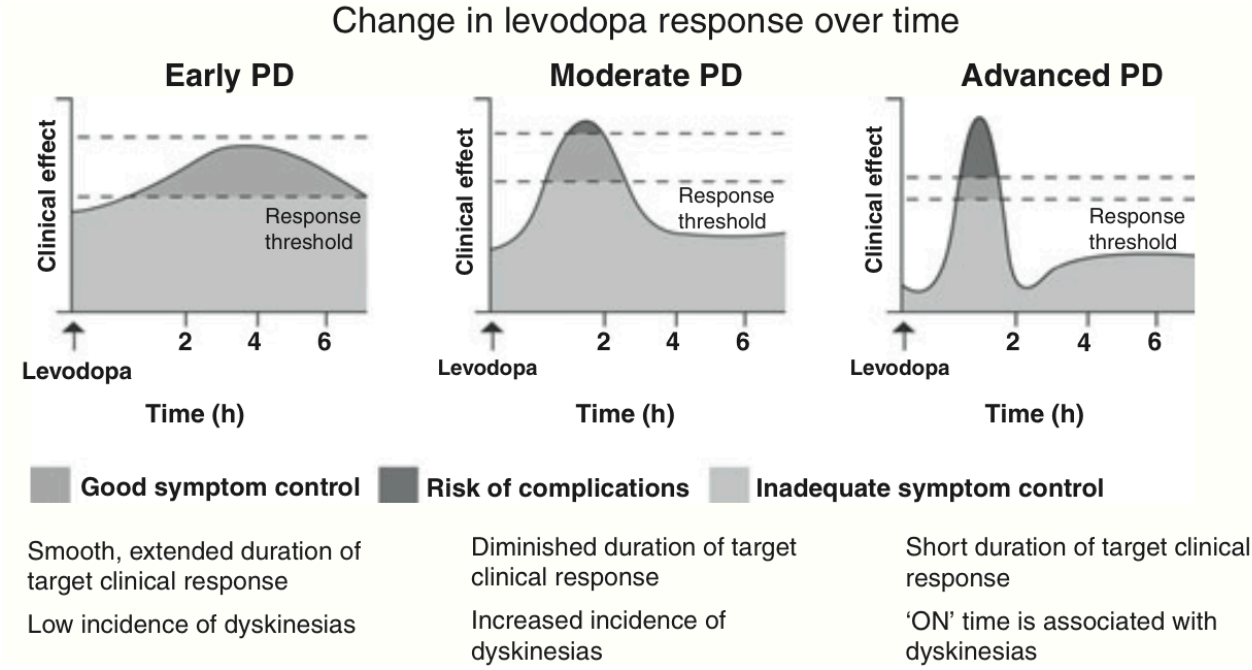

Figure 2: The three graphs show the changes in L-DOPA response with increasing disease duration as well as the advent of complications due to L-DOPA treatment (Schapira et al. 2009).

Motor complications were also noted in the early days of L-DOPA treatment(Cotzias et al. 1969), and affect the majority on chronic L-DOPA treatment(Ahlskog & Muenter 2001). Among these were fluctuations in diurnal response to L-DOPA. Some patients in Cotzias study suffered from the abrupt loss of treatment effect, leaving them hypo- or akinetic for minutes to hours albeit continuing the same treatment regimen. These "ON/OFF"-fluctuations seemed to be related to disease duration more than disease severity. As can be seen in figure 2, this is a well recognized issue related to L-DOPA treatment in advanced PD. Initially the fluctuations are often seen as a "wearing off"-effect or "end of dose"-deterioration, but in more advanced stages fluctuations become more severe with abrupt development of akinesia in an increasingly random pattern. Fluctuations might occur when insufficient dopaminergic neurons remain to create a stable tonic DA stimulation. As discussed in (Andersen *et al.* 2017)(Appendix II) L-DOPA metabolism in other non-dopaminergic neurons might also contribute to these fluctuations, as well as post-synaptic dopamine-receptor changes.

L-DOPA-induced dyskinesia (LID) was also quickly noticed in the early days of L-DOPA treatment, initially described as myclonic, hemiballistic and choreic movements(Cotzias et al. 1969). Roughly 40% of patients will develop some degree of dyskinesia after 4-6 years of L-DOPA-treatment, increasing to around 90% after even longer treatment duration(Ahlskog & Muenter 2001). When LID develops, also called priming, it is irreversible. Risk factor for LID development include younger age at onset, larger L-DOPA-doses, female gender and lower body weight; the last two pointing to an effect of L-DOPA dose per weight (Olanow *et al.* 2013). In general, dyskinesia is divided into *three main categories* (Marsden & Donaldson 2012)(see fig. 3):

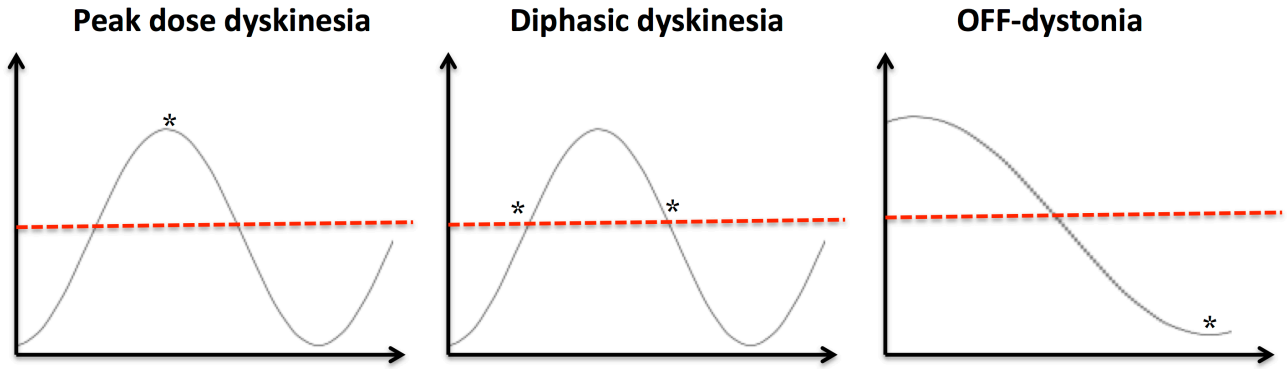

Figure 3: Shows the different types of dyskinesia. The y-axis is the CNS concentration of L-DOPA, the x-axis is time, and the dotted line indicates effect threshold. The asterisk indicates the onset of dyskinesia/dystonia.

Peak-dose dyskinesia: Choreic, ballistic or stereotype movements occurring when the L-DOPA treatment is working most efficiently.

Diphasic dyskinesia: The same movement pattern as peak-dose dyskinesia, but occurring at the beginning and ending of the L-DOPA effect threshold.

Off-dystonia: Often seen in the morning, when L-DOPA-plasma levels are low or zero, with painful dystonia in the feet.

Dystonia might also be seen in diphasic dyskinesia. Peak-dose dyskinesia is not as disabling as diphasic dyskinesia, and patients might not even be aware of their existence. On the other hand dyskinesia can be so severe that it affects or prevents performing many daily activities. As noted, ON/OFF-fluctuations are frequent in L-DOPA treated PD patients, and dyskinesias will successively increase in frequency. Thus, the primary treatment strategy when LID has occured involves adjusting L-DOPA treatment to minimize fluctuations. Stabilizing L-DOPA

plasma levels might be done using the COMT-inhibitor entacapone (Nutt *et al.* 1994) (combined with L-DOPA and benserazid in the drug Stalevo®), decreasing the pulsatile DA distribution pattern due to an increased half life of L-DOPA. This was thought to attenuate LID (Marin *et al.* 2006), but in the study STalevo Reduction In Dyskinesia Evaluation in Parkinson's Disease (STRIDE-PD)(Stocchi *et al.* 2010) conversely patients treated this way actually had an increased risk of LID.

The most frequently used drug against LID is the non-selective N-methyl-D-aspartate (NMDA)-antagonist amantadine, which has been shown to significantly decrease the subjective experience of LID (da Silva-Junior *et al.* 2005) as well as attenuating intravenous L-DOPA induced peak-dose dyskinesia(Metman *et al.* 1998). A study showed a sustained response at 1-year follow up(Metman *et al.* 1999), in contrast to a maximal 8 month effect of amantadine on LID due to oral L-DOPA(Thomas *et al.* 2004).

The continuous intestinal administration of L-DOPA (e.g. Duodopa®) leads to more stable plasma L-DOPA levels(Nyholm *et al.* 2013). It not only seems to decrease OFF-time as compared to immediate release L-DOPA, but also increases patient reported ON-time without troublesome dyskinesia(Olanow *et al.* 2014, Antonini *et al.* 2016). This indicates that pulsatile L-DOPA stimulation contributes to LID, which will be discussed later.

Alternatively, surgical intervention is also possible with a relatively low complication rate(Chen *et al.* 2016). Apart from providing a significant increase in motor function, deep brain stimulation (DBS) of the subthalamic nucleus (STN) or the internal globus pallidus (GPi) also significantly decreases dyskinesia in advanced PD(Esselink *et al.* 2004, Anderson *et al.* 2005, Williams *et al.* 2010). The proposed, and quite complex, pathophysiology behind LID will be discussed in the section "**L-DOPA-induced dyskinesia**".

Dopamine-agonists:

Drugs such as pramipexole and ropinirole work directly on dopamine receptors and do not require conversion to a functional substance. Although significantly less effective than L-DOPA, they are often used in the earlier stages of the disease. Due to a longer half-life they deliver a more sustained therapeutic response. Using DA-agonists before L-DOPA delays the development of dyskinesia. Side effects include nausea, orthostatic hypotension, various types of mental disturbances as well as the risk of impulse control disorders.

Monoamine oxidase-B-inhibitors:

The drugs selegiline and rasagiline provide irreversible MAO-B-selective inhibition, thus decreasing the MAO-B-associated metabolism of DA. Pre-synaptic DA autoreceptors are also blocked, resulting in decreased reuptake of DA. Together, this increases the availability of DA. The drugs can be used in early disease stages, slowing disease progression and delaying the time at which L-DOPA treatment is necessary.

The fact that these drugs, used for more than 40 years, are still the most efficient drugs available is quite remarkable, and there is a continuous need for the development of more efficient treatment options. Essentially the development of disease modifying drugs that might slow down the incessant neurodegeneration in PD is much needed.

2.2.4 Parkinson's disease and neuropathology

Almost a hundred years after James Parkinson's descriptions of the shaking palsy, Friedrich Lewy in 1912 identified cellular inclusions in the neurons of patients with PD. Seven years later, Konstantin Tretiakoff, who coined the term Lewy body (LB), noticed the specific damage to the substantia nigra pars compacta (SNpc) in PD (Holdorff 2002). These cellular inclusion became one of the neuropathological hallmarks of PD(Dickson *et al.* 2009). In 1997 Spillantini et al(Spillantini *et al.* 1997) identified the protein alpha-synuclein (α-syn) as the main constituent of LB. The same year Polymeropoulos et al(Polymeropoulos *et al.* 1997) identified a point mutation in the α-syn gene in a family with hereditary PD, underlining the importance of α-syn in the PD pathophysiology. α-syn will be described in detail later in the thesis.
The progressive character of the disease seems also to be reflected in the neuropathological findings. In a much cited article from 2003, Heiko Braak and colleagues(Braak *et al.* 2003), using α-syn immunohistochemistry, investigated the brains of patients with incidental LB disease. They identified a stereotypical pattern with which α-syn pathology spreads, and proposed 6 stages of LB pathology related to the progression of PD (see fig.4):

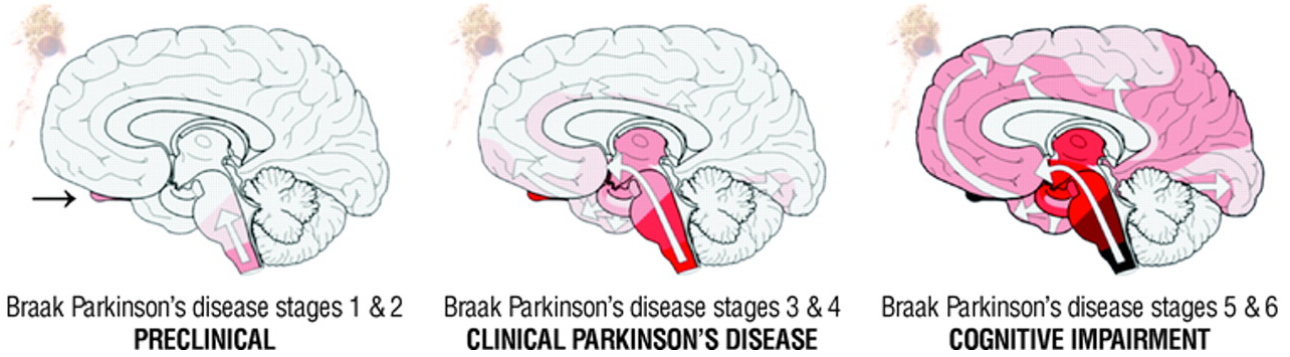

Fig. 4: Progressive Lewy Body (LB) aggregation as proposed by Heiko Braak. Notice the initial involvement of lower brain stem structures and the olfactory bulb, only in later stages involving the substantia nigra pars compacta. LB pathology in cortical regions entails the onset of cognitive impairment (Olanow et al. 2009).

Stages 1 and 2 are thought to be the preclinical stages of neurodegeneration: Initially LB and Lewy neurites (LN) are found in the dorsal glossopharyngeal and vagal motor nucleus (DMV), progressing in stage 2 to involve pontine structures such as the raphe nuclei, the reticular substance and the coeruleus-subcoeruleus complex.

Stages 3 and 4 mark the beginning of the clinical stage of the disease. The occurrence of LB in melanin-laden cells progresses, and mesencephalic affection is now evident in the SNpc. Differing stages 3 and 4 is the initial involvement of mesocortical areas, the macroscopically recognizable depigmentation in the SNpc, and involvement of magnocellular nuclei just ventral of the basal ganglia.

Stages 5 and 6 are characterised by the involvement of neocortical regions. From a severe affection of the olfactory area in stage 5, stage 6 shows affection of almost the entire neocortex.

The proposed staging has been validated in more recent studies(Dickson *et al.* 2010, Kingsbury *et al.* 2010, Parkkinen *et al.* 2008). Conversely one study criticized the use of asymptomatic subjects with LB pathology, since it could be questioned whether these subjects would develop symptoms or PD at all(Kalaitzakis *et al.* 2008). In that study, 47% of the included PD patients did not fit the rostro-caudal progression model, 24% showed no marked DMV affection despite severe SNpc affection, and 7% did not show affection of the DMV despite α-syn aggregation in SNpc(Kalaitzakis et al. 2008). Also, 60% of patients without DMV involvement had α-syn lesions in the spinal chord (SC). This clearly contests the idea of a consistent medullary origin of α-syn pathology in the PD CNS.

Interestingly, progressive α-syn pathology regardless of clinical appearance does not necessarily correlate with extrapyramidal symptoms (as expected when SNpc is affected) or cognitive decline (as expected when cortical α-syn pathology is widespread)(Parkkinen et al. 2008). It might be that LB in itself does not correlate well with cell damage. Affirming this notion, a study(Kalia *et al.* 2015) reviewing several neuropathological studies(Ross *et al.* 2006, Gomez & Ferrer 2010, Gaig *et al.* 2008, Giasson *et al.* 2006, Vitte *et al.* 2010, Poulopoulos *et al.* 2012, Hasegawa *et al.* 2009, Ujiie *et al.* 2012, Wszolek *et al.* 1997, Khan *et al.* 2005, Puschmann *et al.* 2012, Giordana *et al.* 2007, Rajput *et al.* 2006, Goldwurm *et al.* 2006, Ruffmann *et al.* 2012, Gaig *et al.* 2007, Ling *et al.* 2013, Marti-Masso *et al.* 2009) of patients with the hereditary PARK8 mutation of the leucine-rich repeat kinase 2 (LRRK2) gene found that several patients did not have marked LB pathology despite having motor symptoms. It seems, though, that non-motor features such as cognitive impairment, anxiety and orthostatic hypotension were more prevalent in patients with relevant LB pathology(Kalia et al. 2015). In parkin gene (PARK2) related hereditary PD, neuropathological examinations do not show LB accumulation despite nigral degeneration (Doherty *et al.* 2013); these patients suffer from an earlier onset of PD. Maybe LB generation could actually be a resort for removing otherwise damaging proteins when other degradation pathways are insufficient, and maybe the creation of LB is ubiquitine dependent?

A peripheral or central trigger?

Several studies have also focused on peripheral α-syn aggregation in PD, identifying phosphorylated α-syn in submandibular glands(Beach *et al.* 2013), α-syn in salivary glands(Cersosimo *et al.* 2011), intraneuronal phosphorylated α-syn in skin biopsies (Donadio *et al.* 2016), α-syn and LB in the olfactory bulb(Daniel & Hawkes 1992, Beach *et al.* 2009), and intraneuronal α-syn aggregation in the enteric nervous system (ENS) of the gastric mucosa (Sanchez-Ferro *et al.* 2015, Braak *et al.* 2006). REM-sleep behaviour disorder (RSBD) in the setting of a degenerative dementia or Parkinsonism disorder is highly indicative of a synucleinopathy (Boeve *et al.* 2001), and RSBD itself poses a 10 year risk of 75% for developing a neurodegenerative disorder(Iranzo *et al.* 2014). A study found phosphorylated α-syn (p-α-syn) skin deposits (especially in autonomic fibres) in PD patients (sensitivity 80%), in RSBD patients (sensitivity 55.6%), and in no controls (specificity 100%) (Doppler *et*

al. 2017). In RSBD p-α-syn-deposits even correlated with olfactory deficiency and the probability of prodromal PD (Doppler et al. 2017). α-syn can spread from neuron to neuron(Danzer *et al.* 2012, Masuda-Suzukake *et al.* 2014), and it has been hypothesized that synucleinopathies (such as PD, Lewy Body Dementia (DLB) and multiple system atrophy (MSA)) are to a certain extent prion like disorders(George *et al.* 2013, Dunning *et al.* 2013, Ugalde *et al.* 2016). But it does not tell us where protein aggregation occurs initially. Does the detrimental process begin in the PNS, just to propagate to the CNS? Some evidence points to a peripheral trigger: Hyposmia(Ross *et al.* 2008) as well as constipation(Schrag *et al.* 2015) are well-known premotor symptoms of PD, pointing to a potential olfactorial or ENS affection prior to damage of the SNpc. Exposure to environmental toxins such as pesticides significantly increases the risk of developing PD(Gorell *et al.* 1998). Water consumption from private wells also correlates with PD development. These wells are shallower and therefore more likely to be exposed to ground toxins. A well-known PD animal model (Pan-Montojo *et al.* 2012) was made by oral administration of rotenone. This environmental toxin promoted α-syn aggregation in the ENS, with progressing α-syn pathology to the DMV, and vagotomy decreased the severity of α-syn aggregation. In the same paper they found a retrograde transport of α-syn in sympathetic neurons connected to rotenone treated ENS cells, all in all indicating a potential spread of α-syn through sympathetic and parasympathetic fibres due to rotenone exposure (Pan-Montojo et al. 2012). Findings from a Danish register study also seem to corroborate the notion, showing a decreased incidence of PD in truncally vagotomised patients compared to age matched controls (Svensson *et al.* 2015). However, an animal study has shown that after viral vector mediated over-expression of human α-syn in the mesencephalon, α-syn proteins spread to cholinergic fibres in the DMV to preganglionic vagal projections of the myenteric plexus(Ulusoy *et al.* 2016). This could warrant a disease mechanism with central onset, spreading to the PNS through the vagal nerve. Several disease mechanisms are involved in the onset of PD (as will be described later), which could explain the differences found in neuropathological studies. It might be prudent to view PD as a symptomatic diagnosis with several neuropathological pathways, whose only common denominator so far seems to be the marked degeneration of SNpc.

2.3 Biomarkers:

Since this thesis specifically deals with the use of biomarkers, I will try to describe what is actually meant by that term. The National Institutes of Health Director's Initiative on Biomarkers and Surrogate Endpoints chose to define a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"(Biomarkers Definitions Working 2001). Thus, biomarkers can be used for diagnosis, disease staging, disease prognosis indication and monitoring of clinical response (Biomarkers Definitions Working 2001). The degeneration of SNpc is pivotal for PD, and biomarkers that in some way reflect the degeneration of SNpc may be the most optimal *diagnostic* biomarker, but since the disease process are initiated way earlier, it might be seen as a quite late *diagnostic* biomarker. On the other hand, the further away focus is moved from this main feature of SNpc-degeneration, the less certain we are that a biomarker is a common feature for all PD patients or even PD as a disease entity (see fig.8).

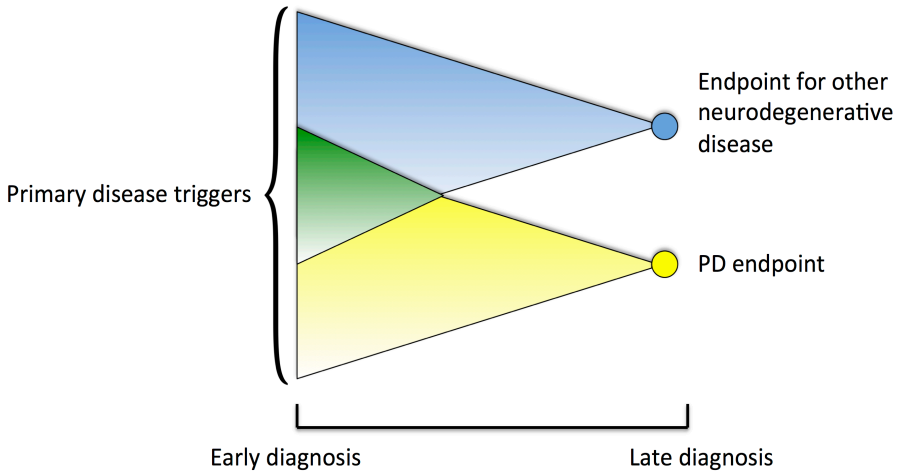

Figure 8: A schematic depiction of the challenges in biomarker research. Early biomarker candidates for PD may be affected in other neurodegenerative diseases (overlap coloured green), and there may be many potential disease triggers. Focusing on early biomarker candidates may require a broader focus, such as metabolomics. The heterogeneous disease processes probably converge to the endpoint. PD; Parkinson's disease.

Also relevant for this study is the use of surrogate endpoints, described as "A biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence"(Biomarkers Definitions Working 2001). This thesis focuses on the effect of L-DOPA on the development of L-DOPA-induced dyskinesia (LID), identifying potential surrogate endpoints for LID. The use of the term "validation" in that context is being questioned(Biomarkers Definitions Working 2001, Strimbu & Tavel 2010). The most uncompromising interpretation of the term validation would imply, e.g. in regards to LID, that LID surrogate biomarkers identified represent all known mechanisms by which L-DOPA is able to induce LID. It is well known, and will be discussed later in this thesis, that LID is a multifactorial complex side effect(Jenner 2008, Huot *et al.* 2013, Bastide *et al.* 2015); as such the term validation, if used e.g. in regards to stating that studies are needed to validate our findings, should be interpreted as an evaluation rather than validation. Probably applicable for both PD diagnostic biomarkers and LID surrogate endpoints is the notion that "the use of multiple biomarkers that represent various components of complex disease pathways"(Biomarkers Definitions Working 2001) will generate a more complete estimate.

Chapter 4: Aims of the study and hypothesis

4.1 Aims:

In this project there were two major aims.

1. To evaluate biomarker candidates for diagnosing Parkinson's disease.
2. To evaluate biomarker candidates representing LID.

Included in these aims is also the idea of further elucidating the complex underlying processes involved in both the disease itself but also LID.

4.2. Hypothesis:

By analysing certain proteins, metabolites and enzyme activities in CSF and blood samples from PD patients, including patients suffering from LID, it is possible to not only differentiate patients from controls, but also to identify dyskinetic PD patients due to specific changes in certain metabolic pathways.

The distinct biomarker studies in this thesis are involved in both of these aims. Instead of individually describing the studies, this thesis subdivides the biomarker results and biomarker discussion section into the two major issues: Diagnosing PD and identifying LID. Thus, the methods section will be a walkthrough of all methods used during the project.
In the results and discussion section I have summarized the most important findings from our articles and manuscript (see appendix I-IV). Some results will be discussed in more detail, since they are not included in the manuscripts (GCase, VEGF, α-syn).

To understand the rationale behind the choice of biomarker candidates in the thesis, it is important to understand the proposed underlying pathophysiological pathways involved in PD and LID. Although, from a pragmatic viewpoint, any biomarker that specifically and sensitively separates PD from controls or identifies LID would be useful, even if the underlying mechanisms were not fully understood. In the following chapter, I will briefly discuss the pathophysiological pathways proposed in PD and in the development of LID as

well as the biomarker studies focusing on these pathways, identified in our systematic review (Andersen et al. 2016)(Appendix I)

Chapter 5: Pathophysiology and current knowledge in biomarker research

5.1 Biomarkers for diagnosing Parkinson's disease:

5.1.1 Preface

"In most cases, especially in those in which the disease has been allowed to exist long unopposed, it may be found that all that art is capable of accomplishing, is that of checking its further progress" – James Parkinson (1817)

Two important aspects with regards to diagnosing PD have to be pointed out:
Firstly, motor symptoms of PD occur late in the disease process. One study found a 31% degeneration of dopaminergic neurons in the SNpc prior to the development of motor symptoms, and following the advent of motor symptoms an exponential cell loss not accounted for by age (Fearnley & Lees 1991). Interestingly it is noted that 80% of DA in the striatum is lost before the patient develops motor symptoms(Marsden 1990), pointing to a dysfunction of the remaining dopaminergic neurons. As mentioned before it has become evident that pre-motor features such as olfactory deficiency, constipation, and RSBD are quite prevalent, pointing to a pre-substantia nigra disease activity of the nervous system in the olfactory bulb, the ENS or the brain stem respectively. Even regional pain symptoms can precede the motor symptoms. Taken together, this means that it is potentially possible to diagnose patients prior to the development of motor symptoms and thus prior to the more pronounced degeneration of dopaminergic neurons.
Secondly, misdiagnosis in a clinical setting is common. A metaanalysis found a 79.6% accuracy of expert clinical diagnosis, rising to 83.9% at follow up, with non-experts performing poorer(Rizzo *et al.* 2016). In the same review atypical Parkinsonian syndromes (APD), such as the synucleinopathies (diseases proposed to be caused by α-syn aggregation) Lewy Body

dementia (DLB) and multiple system atrophy (MSA) as well as the tauopathy (diseases proposed to be caused by tau accumulation) progressive supranuclear palsy (PSP) were often alternative diagnoses(Rizzo et al. 2016). MSA (Ahmed *et al.* 2012) and PSP(Steele *et al.* 2014) have several symptoms that distinguish them from PD especially in later stages such as a more rapid development and little to no effect of L-DOPA treatment. DLB patients can have Parkinsonian motor symptoms but DLB is characterized by early and rapidly evolving dementia due to cortical affection (Mueller *et al.* 2017). Quite arbitrarily the distinction between DLB and Parkinson's disease dementia (PDD) has been made according to the timing of dementia. If patients develop dementia prior to or within a year of developing Parkinsonian motor symptoms, they are diagnosed as having DLB (McKeith 2006). The new diagnostic criteria from the Movement Disorder Society refute this distinction(Postuma et al. 2015), advocating for labelling patients as "PD (dementia with Lewy bodies subtype)".

These two points are important because: If a sufficiently sensitive and specific diagnostic biomarker for PD could be developed, investigating these biomarkers on people with suspected PD premotor symptoms would allow for an early diagnosis and intervention, if disease modifying drugs are developed. This would be essential for the development of disease modifying treatments. And if the biomarker candidates even showed specificity towards PD compared to APD, misdiagnosis could be limited. In this thesis we have not included any patients with APD, except from one patient suffering from DLB. Therefore the issue of misdiagnosis will not be further elucidated in this thesis, but discussed in the closing remarks.

In our systematic review(Andersen et al. 2016) we found that CSF biomarker studies focused on the main aspects neurotransmitters, oxidative stress markers, inflammatory markers and proteins involved in PD pathology. The diversity of biomarker candidates clearly reflects that PD is a multifactorial complex disorder with many potential pathogenic pathways.

Initially, I will provide a brief overview of known pathological pathways. Pathways related to biomarker candidates involved in this thesis will be described in more detail.; either in this section or in appendix I-IV.

5.1.2 α-synuclein:

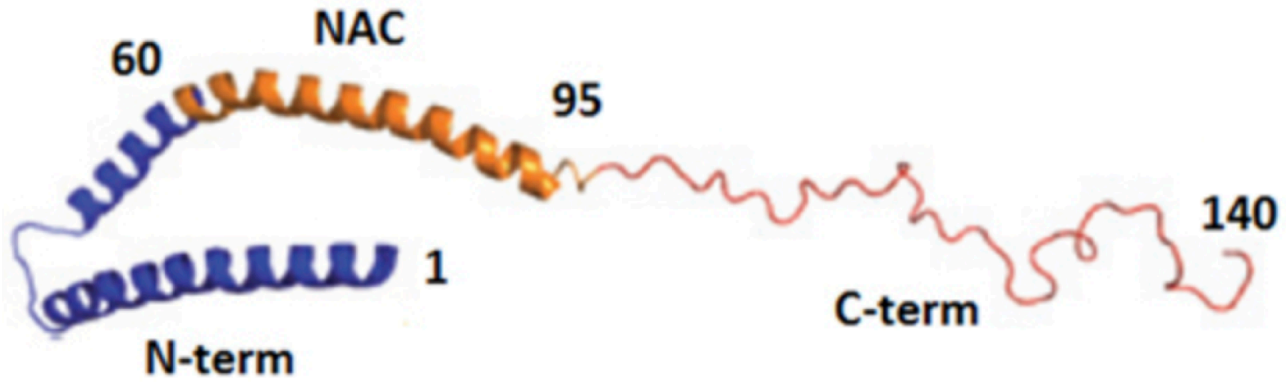

Figure 5: A schematic representation of the alpha-synuclein protein. The N-terminal is marked in blue, the non-Amyloid β-component (NAC) colored orange and the C-terminal colored red. Alpha-synuclein proteins phosphorylated at the serine 129 residue make up a large part of Lewy Bodies, and the phosphorylation takes place in the C-terminal. From (Gallegos et al. 2015).

This 140 aminoacid protein seems to link together several putative PD disease pathways such as mitochondrial dysfunction, lysosomal and proteasomal dysfunction, oxidative stress, and dopamine toxicity, as discussed in reviews (Moore *et al.* 2005, Dev *et al.* 2003). The protein is present in presynaptic terminals. Its functions are related to synaptic plasticity as well regulating vesicular trafficking(Outeiro & Lindquist 2003), and it seems to regulate presynaptic DA release(Larsen *et al.* 2006). Mutations in the α-syn gene are related to familial PD (PARK1 & PARK 4)(Polymeropoulos et al. 1997, Singleton *et al.* 2003). Overexpression of α-syn and the subsequent conformational changes such as oligomerization of α-syn are related to the PD pathogenesis. It is shown to inhibit macroautophagy (Winslow *et al.* 2010), induce lipid and ubiquitin accumulation, and decrease cell growth and proteasomal activity (Outeiro & Lindquist 2003, Snyder *et al.* 2003). It also induces oxidative stress and decreases mitochondrial activity (Hsu *et al.* 2000). Subspecies of oligomeric α-syn also increase Ca^{2+}-influx potentially inducing inositol 3-phosphate (IP_3) related apoptosis(Danzer *et al.* 2007, Angelova *et al.* 2016).

Posttranslational modifications of α-syn might play a role in the pathogenesis: Lewy bodies consist greatly of phosphorylated α-syn (e.g. phosphorylated serine 129)(Fujiwara *et al.*

2002) as well as nitrated α-syn(Giasson *et al.* 2000), and nitrosative stress has been shown to induce aggregation of nitrated α-syn(Paxinou *et al.* 2001).
Results of α-syn biomarker studies are discussed in detail in both our review(Andersen et al. 2016)(Appendix I) and the L-Tryptophanol (Trol) manuscript (Appendix IV).

5.1.3 The complex interplay between oxidative stress, mitochondrial dysfunction and proteasomal dysfunction:

CNS neurons have a very high metabolic rate, and therefore also a high production of reactive oxygen species (ROS) due to the synaptic connectivity and recovery from glutamate induced action potentials as well as mitochondrial activity (Attwell & Laughlin 2001). Especially dopaminergic neurons are intrinsically exposed to higher levels of ROS due to DA metabolism (discussed in detail in appendix II (Andersen et al. 2017)). Neurons are provided with high amounts of glutathione (GSH) precursors by astrocytes(Dringen *et al.* 2000), that also contain high levels of vitamin E (Makar *et al.* 1994) with the ability to prevent ROS from ravaging the cell through lipid peroxidation, DNA damage etc.
Our review (Appendix I, (Andersen et al. 2016)) found that CSF biomarker studies focusing on oxidative stress revealed differences in PD CSF compared to controls. This includes increased lipid peroxidation, increased concentrations of nitrates, and decreased coeruloplasmin ferroxidase and Cu/Zn-superoxide dismutase activity(Boll *et al.* 2008). Also a more rapid production of advanced oxidized protein products (Garcia-Moreno *et al.* 2013), decreased glutathione S-transferase Pi activity(Maarouf *et al.* 2012), increased levels of nitrotyrosine products(Fernandez *et al.* 2013), and 8-hydroxyguanosine(Abe *et al.* 2003, Kikuchi *et al.* 2002) has been found in PD CSF. DJ-1 functions as a "sensor of oxidative stress", protects the cell from oxidative stress by acting as a ROS scavenger (Xu & Moller 2010), protects mitochondria from oxidative stress (Hao *et al.* 2010), and in its slightly oxidized state prevents α-syn fibrillation by working as a redox-responsive molecular chaperone (Zhou *et al.* 2006). Mutations in the DJ-1 gene are related to autosomal recessively inherited early onset PD (PARK7)(Bonifati *et al.* 2003). A decrease in DJ-1 would mitigate oxidative stress, and a decrease of DJ-1 has been found in PD CSF(Hong *et al.* 2010). Interestingly it seems that oxidative stress may be more pronounced in early PD, as discussed in our review. Maybe the initially well functioning cellular machinery (and thus a high production of ROS), combined

with the PD trigger mechanism (such as oligomeric α-syn) results in higher levels of oxidative stress in early PD.

Parkin is a ubiquitin ligase involved in ubiquitin-dependent proteasomal activity. Mutations on the Parkin gene (PARK2) is related to autosomal recessively inherited early onset PD (Kitada *et al.* 1998). Oxidative stress causes Parkin misfolding (Winklhofer *et al.* 2003) which affects normal proteasomal functioning.

To link oxidative stress and mitochondrial function, I will initially mention the interplay between Parkin and the phosphatase and tensin homolog-induced putative kinase-1 (PINK1). PINK1-mutations are related to autosomal recessively inherited PD (PARK6)(Valente *et al.* 2004). PINK1 is a mitochondrial kinase that recruits Parkin, marking damaged mitochondria for destruction (mitophagy)(Narendra *et al.* 2010), thereby maintaining a high mitochondrial quality (Pickrell & Youle 2015). Decreased mitophagy would decrease mitochondrial function, which can lead oxidative stress, decreased proteasomal function and α-syn aggregation (Ryan *et al.* 2015). Decreased mitochondrial functioning due to complex-I inhibition of the electron transfer chain is a well known effect of toxins such as paraquat, MPTP and rotenone, that are all related to the development of PD, selectively damaging dopaminergic neurons(Bove *et al.* 2005).

5.1.4 Lysosomal dysfunction:

In the most common autosomal recessively inherited lysosomal storage disease, Gaucher's disease (GD), a mutation occurs in the glucocerebrosidase gene (GBA1) coding for the enzyme glucocerebrosidase(Hruska *et al.* 2008). Symptoms generally include hepatosplenomegaly, thrombocytopenia, bone disease and tendency of bleeding(Thomas *et al.* 2014), but the clinical presentation is very heterogeneous in terms of symptoms and age at disease onset with over 200 known existing mutations in the GBA1 gene(Hruska et al. 2008). A general distinction can be made between three types of GD:

Type I (non-neuropathic) generally involves the aforementioned organ and bone affections (Thomas et al. 2014), whereas **type II** (acute neuropathic) manifests in early infancy with many symptoms like type I but also involving aggressive neurodegeneration (bulbar affection, opisthotonus, seizures) with lethal outcome within the first years of life. **Type III** (neuropatic) does cause in neurological symptoms, such as slowing of horizontal eye movements,

myoclonic epilepsy and sometimes cognitive impairment, but neurodegeneration is less aggressive compared to type II with patients surviving into their third or fourth decade(Erikson *et al.* 1997).

For more than a decade(Aflaki *et al.* 2017), a correlation between GD and PD has been noted, and it has been established that GBA1 mutations (especially a missense mutation replacing leucine in residue 483 with proline (L444P) and a missense mutation replacing asparagine in residue 409 with serine (N370S)(Ortiz-Cabrera *et al.* 2016)) represent the most significant genetic risk factor for the development of PD (odds ratio >5 compared to controls)(Sidransky *et al.* 2009). Increased risk of PD in healthy relatives to GD patients exists, also pointing to a risk of PD development in carriers of GBA1 mutations(Goker-Alpan *et al.* 2004). Parkinsonian manifestations due to GBA mutations are associated with a more rapid disease progression and dementia development(Winder-Rhodes *et al.* 2013). In regards to the earlier development of dementia, it has been shown that DLB is even more significantly related to GBA1 mutations, and that DLB patients with GBA1 mutations also suffer from an earlier onset of cognitive dysfunction(Nalls *et al.* 2013).

One of the substrates for the glucocerebrosidase enzyme (GCase) is α-syn, and it has been proposed that the risk of developing PD is linked to the dysfunctional clearance of α-syn. It has been shown *in vivo* in mice that inhibiting glucocerebrosidase activity with conduritol B epoxide increases the α-syn immunoreactivity in SNpc compared to untreated controls(Manning-Bog *et al.* 2009). In an elaborate study (Mazzulli *et al.* 2011) the potential link between GCase and α-syn was elucidated. In the lysosome, GCase cleaves the glycosphingolipid glucocerebroside(Brady *et al.* 1965). It was found that decreased GCase activity in neurons both increases the amount of α-syn and glucocerebroside(Mazzulli et al. 2011). Subsequently glucocerebrosides aggregate into tubules that provided a scaffold for the formation and stabilization of oligomeric α-syn species. Furthermore oligomeric α-syn species effectively decreased the amount of GCase released from the endoplasmatic reticulum to the lysosomes, negatively affecting the function of GCase, and creating a potentially vicious circle. In human neuroblastoma cell lines (SH-SY5Y) mutations in PINK1 (PARK6) or overexpression of α-syn also leads to decreased GCase activity(Gegg *et al.* 2012).

A study using induced pluripotent stem cells (iPSC) from patients with GBA1 mutations and GD patients compared to control cells showed an increase in α-syn content, a decreased autophagosome-lysosomal fusion as well as a disrupted calcium homeostasis(Schondorf *et al.*

2014); the latter hypothetically linked to the effect of increased levels of o-α-syn(Danzer et al. 2007).

5.1.5 Growth factors:

Several growth factor hormones might play a role in PD. An increase of brain derived neurotrophic factor (BDNF) levels was found in PD CSF(Zhang *et al.* 2008), maybe as a reaction to the decrease of striatal DA, since BNDF upregulates D1 receptor production (Do *et al.* 2007). Vascular endothelial growth factor (VEGF) is a hormone regulating angiogenesis(Leung *et al.* 1989) by increasing endothelial proliferation(Pedram *et al.* 1998), migration(Barleon *et al.* 1996), and survival(Gerber *et al.* 1998), playing a critical role during embryonic development(Miquerol *et al.* 2000). VEGF and other markers of angiogenesis have been found to be upregulated in PD CSF(Janelidze *et al.* 2015), and VEGF has been shown to protect against dopaminergic neuron damage, possibly due to its effect on angiogenesis and glial activation (Yasuhara *et al.* 2005). It could be speculated that a combined stimulation of angiogenesis and neurogenesis through VEGF- and BDNF-receptors is an adaptive response to neuronal damage in the substantia nigra.

4.1.6 Monoamines:

Due to the marked damage to dopaminergic neurons, many researchers, focusing especially on changes in DA and DA metabolites, have performed CSF monoamine analyses. A detailed walkthrough of these findings was done in our catecholamine article(Andersen et al. 2017) (Appendix II).

5.2 Biomarkers for L-DOPA induced dyskinesia:

5.2.1 Preface

As for diagnostic biomarkers, we need an understanding of the pathways leading to LID, in order to develop viable biomarker candidates for LID. Few CSF biomarker studies have been made, actively distinguishing dyskinetic from non-dyskinetic PD; and it does pose a challenge: The development of LID is highly complex, involving changes in several neurotransmitter pathways (Huot et al. 2013, Bastide et al. 2015), but LID is a dynamic process potentially requiring CSF studies to be performed at a moment where dyskinesia is ongoing. Some important pathways related to the project are briefly discussed below:

5.2.2 The dopaminergic system:

The simplest model of dyskinesia involves the D1 DA receptor (see fig.6). It was proposed that direct pathway stimulation through D1 receptors in the striatum would inhibit the internal globus pallidus (GPi), thereby increasing thalamic activity in the ventrolateral and centromedian nucleus, resulting in activity of the supplementary motor cortex and LID (DeLong 1990). D1 activity has been shown to relate to dyskinesia in an animal study (Aubert *et al.* 2005), but the priming event might be dependent on both D1- and D2-receptors (Huot et al. 2013), the two most frequent DA receptors in the striatum. It seems that a decreased internalisation of D1-receptors due to decreased striatal proteasomal activity (20s proteasome) caused by L-DOPA stimulation of D1-receptors in a dopamine-depleted striatum is correlated to development of LID(Berthet *et al.* 2012). This creates an interesting link between α-syn aggregation, oxidative stress and LID, since both affect the proteasomal system. Thus, increasing doses of α-syn in its monomeric state inhibits 20s proteasomal activity(Snyder et al. 2003). Interestingly, patients with Parkin mutations, affecting proteasomal functioning, are more prone to LID development(Kitada et al. 1998).

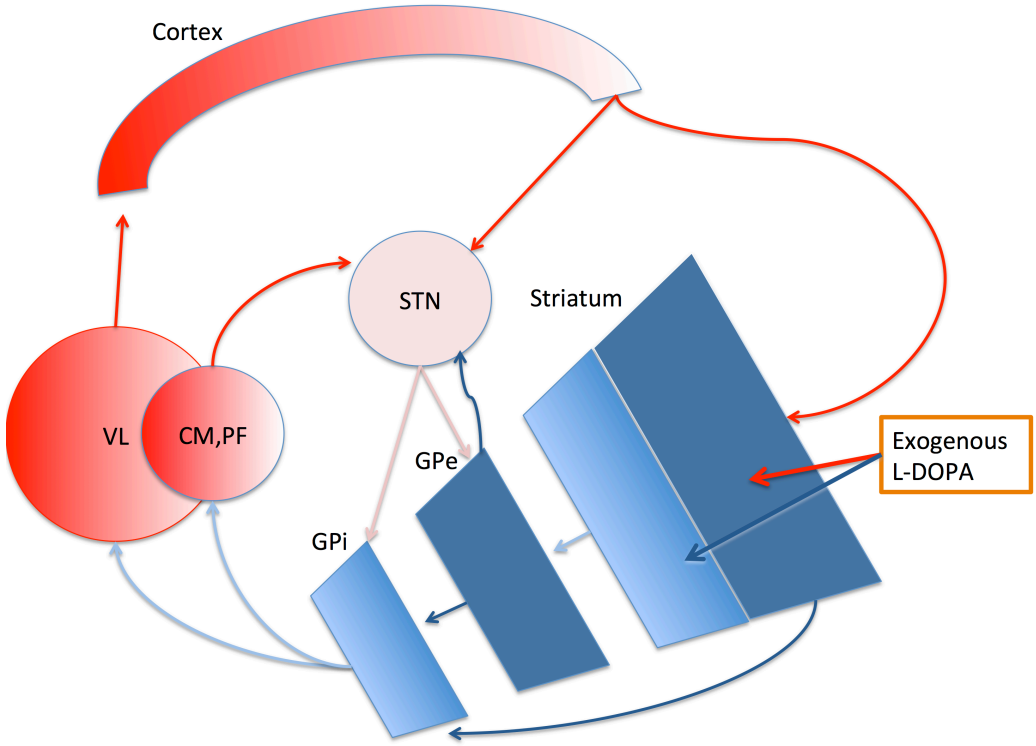

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Figure 6: Adapted from Bastide et al, 2015. This is a schematic presentation of the simplest dyskinesia model. Blue colors indicate inhibitory effects, dark blue indicates higher activity, lighter blue lower activity. Red indicates excitatory effects, dark red indicates higher activity, pink lower activity. It is thought that exogenous L-DOPA affects parts of striatum differently. The medium spiny neurons (MSN) of the indirect pathway (striatum-globus pallidus externus (GPe) - globus pallidus internus (GPi) – thalamus - cortex) are thought to be inhibited by the activation of D2-receptors, whereas MSN of the direct pathway (striatum-globus pallidus internus-thalamus-cortex) are activated by D1-receptor stimulation. Increased GPe activity also inhibites the subthalamic nucleus (STN) otherwise stimulating both GPe and GPi. The effects being a marked decrease in GPi activity due to direct inhibition from striatum as well as inhibition from GPe and decreased stimulation from STN. Decreased GPi activity dis-inhibits activity in the thalamic nuclei (VL: ventral lateral thalamus, CM: Centromedian nucleus, PF: Parafascicular nucleus) increasing neuroexcitation in the supplementary motor cortex.

5.2.3 The glutamatergic system:

Glutamate receptors are highly involved in synaptic plasticity and memory function (Debanne *et al.* 2003), potentially involving these receptors in LID development, since LID is due to a plastic change in neuronal communication. A dopamine modulated increased resting state connectivity between the putamen and the primary sensory-motor cortex and supplementary

motor area is highly predictive of LID development and severity respectively (Herz *et al.* 2015, Herz *et al.* 2016). Previously mentioned, the NMDA-receptor antagonist Amantadine alleviates LID, clearly linking LID and the glutamatergic system. It has been argued that a pulsatile DA release combined with glutamatergic stimulation might induce long term potentiation (LTP), explaining these DA modulated plastic changes(Bastide et al. 2015), and using the radioligand ^{11}C-raclopride it has been shown that acute DA release after L-DOPA intake is more pulsatile in dyskinetic patients (de la Fuente-Fernandez *et al.* 2004). Linking the metabolism of the essential amino acid tryptophane to glutamatergic function and LID, the tryptophane metabolite kynurenic acid (KYNA) has an inhibitory effect on the glutamate N-methyl-D-aspartate (NMDA) receptor(Lanthorn *et al.* 1984). Increasing KYNA production significantly decreases LID in non-human primates (Gregoire *et al.* 2008). Further details on KYNA and the involvement of the tryptophane metabolites 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) are discussed in our article (Havelund *et al.* 2017) (Appendix III).

5.2.4 The serotonergic system:

Serotonergic neurons are important regulators of both dopamine (Olijslagers *et al.* 2006) and glutamate signalling (Lesch & Waider 2012). Due to aromatic L-amino-acid decarboxylase activity in serotonergic raphestriatal neurons(Arai *et al.* 1996), they are able to produce dopamine from L-DOPA. Raphestriatal serotonergic neurons increase L-DOPA-turnover due to nigrostriatal degeneration, potentially being a compensatory mechanism to ensure sufficient DA in the striatum (Carta *et al.* 2007). DA production in serotonergic neurons plays a significant part in LID, and it has been argued that a lack of autoregulatory feedback in serotonergic neurons may cause an excessive DA release (Carta et al. 2007). An increased CSF homovanilic acid (HVA)/DA ratio found in dyskinetic PD patients may support that notion(Lunardi *et al.* 2009), since dopaminergic neurons cannot produce HVA. This differs from our own results, which will be discussed later. Serotonergic neurons may also be involved in the LID priming. Stimulation of the inhibitory 5-hydroxytryptamine receptor 5-HT1 decreases serotonergic DA release (Eskow *et al.* 2009), and 5-HT1A/B receptor stimulation prevents priming (Munoz *et al.* 2008).

5.2.5 Cyclic adenosine monophosphate activity:

Linking dopamine and glutamate receptor in terms of LID development is cyclic adenosine monophosphate (cAMP). A lack of D1 receptor internalization or increased D1 mRNA production due to previously mentioned reasons, could increase DA mediated D1 receptor activity, e.g. both in the striatum but also in cortical regions. The effect would be a downstream production of cAMP, activating the dopamine and cAMP-regulated phosphoprotein of 32 kDA (DARPP-32), which has been shown to be involved in LID(Aubert et al. 2005). This in turn leads to phosphorylation of the glutamatergic receptor GluR1 and phosphorylation of the extracellular signal-regulated protein kinase 1 and 2 (ERK1/2). Phosphorylated ERK (p-ERK1/2) activates the mammalian target of rapamycin (mTORC1), which plays a role in regulating protein synthesis and synaptic plasticity (Costa-Mattioli *et al.* 2009). The net result could be a decreased ability for long term depression and depotentiation leading to LID. The key role of mTORC1 is underlined by the fact that a rapamycin ester (an mTORC1 inhibitor) significantly decreased LID and prevented priming (Decressac & Bjorklund 2013).

5.2.6 Growth factors and plasticity:

VEGF is also of interest with regards to LID. In one elaborate study (Ohlin *et al.* 2011) it was argued that L-DOPA-induced changes in plasticity not only affect neurons but also the microvasculature. They found neuropathological evidence of increased endothelial proliferation in the putamen of dyskinetic PD patients, correlating with VEGF expression. An animal study in the same paper showed L-DOPA to induce angiogenesis and the expression of VEGF as well as an antidyskinetic effect of the VEGF receptor antagonist vandetanib. It has been hypothesized that an affected BBB might be involved in the process (Ohlin et al. 2011), and a correlation between VEGF and BBB leakage has been shown(Janelidze et al. 2015). As stated previously though, VEGF might also have a neuroprotective effect on DA neurons (Yasuhara et al. 2005). VEGF stimulation of endothelial cells also triggers the secretion of BDNF(Louissaint *et al.* 2002). Specific polymorphisms of BDNF are shown to decrease the time for LID development(Foltynie *et al.* 2009), and BDNF also upregulates the D1 receptor(Do et al. 2007) potentially involved in LID development.

Chapter 6: Materials and methods

6.1 Patient recruitment:

Before the initial recruitment procedures, the ethical committee of the Region of Southern Denmark was contacted and gave permission to the execution of the study (s-20130098). Later supplementary applications including the use of blood analyses and new analyses were made and accepted.

For this thesis we planned the inclusion of at least 30 PD patients from the Dept. of Neurology, Sygehus Sønderjylland. The primary contact person at the hospital (Harald Floer, Helle Østerballe) identified suitable patients for the project and initially informed them about the project during their regular visit to the outpatient clinic. If they were interested, further information, both written and oral, was given. A written and oral consent was given before entering the study.

Inclusion criteria included fulfilling the UKBBC(Gibb & Lees 1988), and no patients with suspected APD were included; furthermore patients with severe dementia, severe clinical depression or psychosis, DBS treated and patients with contraindications for performing lumbar puncture (LP) were excluded.

Patient recruitment began late 2014, with the first patient being seen in October 2014. Although many patients were informed about the project only few would accept entering the study. Many were deterred by the lumbar puncture procedure involved. By 2015 it was clear that too few patients were being recruited. This was solved in two ways:

1. We now accepted including patients who would only give permission for taking blood samples for analysis.
2. We broadened our recruitment area.

Regarding point 2; we got permission from the ethical committee to include patients from other centres. Patients were recruited from the private neurological practice of Michael Binzer in Esbjerg, as well as from the neurological depts. of Odense (assisted by Morten Blaabjerg and Matthias Bode) and Roskilde (assisted by Helle Thagesen, and Akram Kamal). From September 2015 to May 2016, an additional 20 patients were included.

6.2 Sampling procedure:

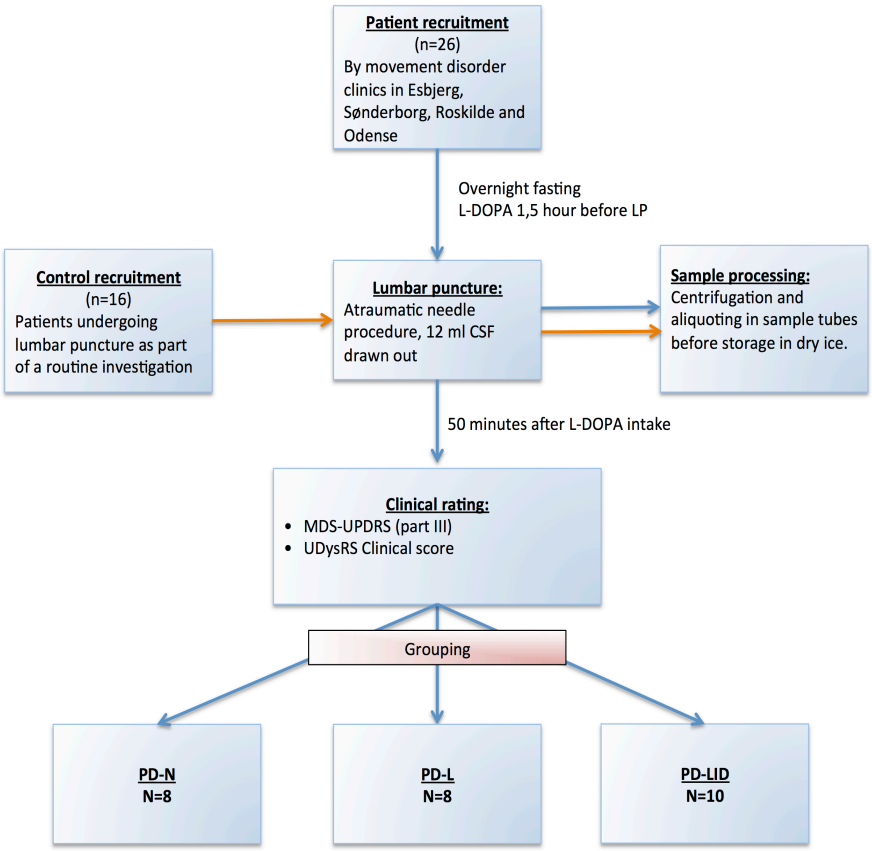

Figure 7: Workflow for patients and controls in the study. All patients and controls were seen on one day each. n;number, L-DOPA; Levodopa, CSF; Cerebrospinal fluid, MDS-UPDRS; Movement Disorder Society Unified Parkinson's Disease Rating Scale; UDysRS; Unified Dyskinesia Rating Scale. Not mentioned in this figure under Clinical Rating is: Montreal Cognitive Assessment (MOCA) and MiniMental State Examination (MMSE).

A quick overview of the process can be seen in figure 7. One workday was assigned for each patient. The patients were examined at the Dept. of Neurology in Sønderborg, Odense, and Roskilde as well as Skleroseklinikken at Esbjerg Sygehus. At 09:30 am the lumbar puncture and blood sampling was performed on the fasting patient. For patients treated with L-DOPA, they were asked to take their morning dose at 08:00 am on the day of examination when possible. This was done primarily because of the catecholamine analyses planned for this study, since L-DOPA significantly alters the concentration of L-DOPA and DA.

The lumbar puncture procedure was done with the assistance of nurses Helle Østerballe and Susanne Aabling (Sønderborg), nurse Rikke Bjerre Rosengren (Esbjerg), nurse Lene Schroll

Kromann and social and health care worker Yrsa Ragnhild Clausen (Odense), and nurse Dea Ledstrup Meyer (Roskilde).

Lumbar puncture was done as described in our published article (Appendix II)(Andersen et al. 2017). Through cooperation with the local clinical biochemical departments in the hospitals of Sønderborg, Esjberg, Odense and Roskilde, routine analysis of CSF was performed. As described in appendix II, some CSF was directly aliquoted and frozen on dry ice for catecholamine analysis. The remaining CSF was kept in ice water until centrifuging.

Blood samples were taking in the decubital fossa using citrate tubes as described in the appendix III (Havelund et al. 2017).

After CSF and blood sampling, samples were centrifuged. CSF was kept in ice water prior to centrifugation whereas blood samples were kept at room temperature (RT). The aim was to centrifuge samples as quickly as possible (within 30 minutes).

CSF was centrifuged at 2000 g, 4° Celsius for 10 minutes before being aliquoted into Saarstedt tubes in 200 microliter and put on dry ice. Before aliquoting CSF, a subset of tubes was prepared with a 75 µL mix of protease and phosphatase inhibitors. One tablet of cOmplete™ mini EDTA-free (catalog no. 11836153001, Roche Applied Science, Indianapolis, USA) and PhosSTOP™ (catalog no. 04906837001) were mixed in 2 ml Phosphate buffered saline (PBS)(Gibco). In these tubes 300 microliter CSF was added.

Either the centrifuge was then heated to RT or a second centrifuge was used. Blood samples (citrate tubes) were then centrifuged at RT, 1400 g for 10 minutes, dividing blood samples into plasma and blood pellets.

Plasma was separated from the blood pellets and centrifuged at RT, 3000 g for 10 minutes before being aliquoted into Saarsted tubes in 200 microliter portions and put on dry ice.

Erythrocyte pellets were rinsed 1:1 in 0,9% NaCl and centrifuged at 3000 g, 4° Celsius for 10 minutes. The supernatant NaCl was removed and the rinsing procedure performed twice more. After the final centrifugation the supernatant NaCl was removed and blood pellets were suspended 1:1 in 0,9% NaCl before being aliquoted into Saarsted tubes in 400 microliter portions and put on dry ice.

The same day, samples were transferred from dry ice to a -80° Celsius freezer (Institute of Molecular Medicine, Neurobiological research).

6.3 Patient examination:

After sample processing, patients were physically examined. For patients taking L-DOPA the clinical rating was done 50 minutes after L-DOPA intake. Two clinical rating scales were used that I will now describe in more detail:

6.3.1 The Movement Disorder Society-Sponsored Revision of the Unified Parkinson's disease Rating Scale (MDS-UPDRS):

The MDS-UPDRS is a clinimetric tool for the assessment of Parkinson's disease severity. It has been validated on a large ethnically varied PD population(Goetz *et al.* 2008b) and has been translated into 15 languages (http://www.movementdisorders.org/MDS/Education/Rating-Scales.htm). A Danish version is currently in the process of validation. The rating scale tools include questions asked by the clinician as well a questionnaire that can be filled out by the patient and/or a caregiver as well as a clinical examination. For the use of the UPDRS Part III rating scale, I have completed an online MDS-UPDRS Training Program and Certificate Exercise (appendix V) (http://www.movementdisorders.org/MDS/Education/Rating-Scales/Training-Programs.htm).

The questions asked focus on the average symptom severity during the last week before the rating. The severity score follows the same principle throughout the entire rating scale:

0 = normal,

1 = slight, used when symptoms are just appreciated by the patient/clinician, but not interfering with performance in any way.

2=mild, more evident symptoms, but only with mild affection appreciated by patient/clinician.

3=moderate, when symptoms are clearly appreciated by patient/clinician, but do not preclude the patient's ability to carry out normal activities or perform motor task as viewed by rater.

4=severe, when symptoms preclude carrying out normal activities or performing a motor task as viewed by clinician.

The entire rating scale is divided into four parts:

Part I:

Focuses on non-motor experiences of daily living, probing into known non-motor issues such as cognitive impairment, hallucinations, depressed mood and autonomic disturbances.

Part II:

Focuses on motor experiences of daily living, giving an insight into the patient's perceived disability related to talking, eating, walking etc.

Part III:

This part is the clinical examination of the patient. This part of the MDS-UPDRS is used in this thesis and will be described in more detail:

Initially it was noted whether the patient is in the ON- or OFF-stage, which will clearly affect the motor abilities of the patient. The use of anti-Parkinsonian medication and the time since last dosage of L-DOPA (when applicable) was noted. After evaluating speech and facial expression, rigidity in all extremities and the neck was rated. Bradykinesia was rated in upper extremities by finger tapping, hand movements and pronation-supination movements of the hands. In the lower extremities toe tapping and leg agility tests rated bradykinesia. Each extremity was examined separately focusing on the "speed, amplitude, hesitations and decrementing amplitude"(Goetz et al. 2008b) of the movements. Arising from a chair, gait, freezing of gait, postural stability, posture, global spontaneity of movement was rated. The global spontaneity was evaluated as an overall assessment of bradykinesia during the entire rating process. Tremor assessment then focused on postural, kinetic and resting tremor, and any dyskinesia and its impact on the rating was noted.

Part IV questions the patient about motor complications related to treatment including dyskinesia, dystonia, and ON/OFF-motor fluctuations.

We translated the questions and questionnaires from part I, II and IV into Danish, since no Danish version existed at the beginning of this project. This was only done from English to Danish. All patients completed parts I, II and IV in this rating scale. Since the requirements from the Movement Disorder Society for validating a translation of the MDS-UPDRS were not met and are quite time consuming(Goetz *et al.* 2014), it was impossible for us to validate the translation in the time frame of this project. I have argued that the UPDRS part III does not include a language barrier since it is purely based on movements, and since I was able to use

the original version for the completion of the patient examination, the results from the UPDRS part III motor score are used in this thesis.

6.3.2 The Unified Dyskinesia Rating Scale (UDysRS):

Goetz et al (2008) argued that a scale featuring the assessment of "anatomical distribution, phenomenology, duration, intensity, disability and patient perceptions"(Goetz *et al.* 2008a) was needed, resulting in the creation of UDysRS. As with the MDS-UPDRS the rating combines questions asked by the clinicians, a questionnaire and a clinical rating. No Danish version was available, and questions as well as questionnaires were translated into Danish. Ratings are also the same integers ranging from 0-4. The rating scale has not been validated on as large a patient population as the MDS-UPDRS, but the intra- and inter-rater reliability for the objective assessment was deemed acceptable(Goetz et al. 2008a). An online MDS-UDysRS Training Program and Certificate Exercise has been completed for the use of the objective assessment (http://www.movementdisorders.org/MDS/Education/Rating-Scales/Training-Programs.htm) (appendix VI).

The rating scale consists of four parts, two historical parts and two objective parts:
Historical:
Part I rates historical ON-dyskinesia. Initially it is quantified how much of the day a patient suffers from these medicine-induced dyskinesias. Afterwards a questionnaire focuses on the effect of ON-dyskinesia on several activities of daily living (ADL).
Part II rates historical OFF-dystonia.
Objective:
The objective dyskinesia assessment centres upon four tasks representing ADL, recorded on video for later rating (Rush Filming Protocol).
Task 1, communication: The patient sits in a chair, describing a drawing that he/she is holding with both hands.
Task 2, drinking from a cup: The patient sits in a chair, graps a cup with water, takes a sip and puts down the cup again; repeats.
Task 3, dressing: The patient puts on a lab coat, buttons three buttons, unbuttons and takes off the lab coat.

Task 4, ambulation: The patient rises from a chair, walks 5 meters, turns, walks back to the chair and sits.

Part III rates impairment due to dyskinesia. For each task described above, the severity of dyskinesia is rated separately in the face, neck, extremities and trunk. A final score for the face, neck, extremities and trunk will be the most severe score recorded in any of the four tasks.

Part IV rates disability due to dyskinesia. Instead of focusing on specific body parts this score focuses on the overall effect of the total amount of dyskinesia on a specific task.

It is also noted whether the patient exhibits ON-dyskinesia, OFF-dystonia (or transition state), whether chorea and/or dystonia was present, as well as which one was predominant of the two.

A total objective score is found by adding ratings from part III and IV.

As with the MDS-UPDRS our Danish translation of the questions and questionnaire of the UDysRS was not validated, and as such cannot be used for further analysis. I have argued that scores from parts III and IV are usable for further analyses, since I was able to use the original paper for performing the rating.

It is to be noted that permission to use these specific parts of the MDS-UPDRS and UDysRS has been granted by the Movement Disorder Society (appendix VII).

6.3.3 Cognitive rating:

Patients were evaluated using Danish versions of both the Mini-Mental State Examination (MMSE) (https://www.sundhed.dk/sundhedsfaglig/laegehaandbogen/undersoegelser-og-proever/skemaer/geriatri/mms-mini-mental-status/) and the Montreal Cognitive Assessment (MOCA) (http://www.mocatest.org/wp-content/uploads/2015/tests-instructions/MoCA-Test-Danish-2010.pdf). Especially the MOCA test has been proven to perform well as a screening tool for PD patients(Gill *et al.* 2008).

6.3.4 Health information:

Disease duration in this thesis is defined as the time from the patient noted the first symptoms of Parkinson's disease to the date of examination in this project. Duration since receiving the diagnosis by a professional was also noted.
Information about other illnesses, medication, hereditary diseases was noted.
Patients were grouped according to the use of L-DOPA and the presence/absence of LID. One patient had a clear dystonic dyskinesia, but was included prior to the use of the UDysRS, and one patient described daily symptoms interpreted as being dyskinetic, but these were not evident at examination. As can be seen in table 2, we have included a variable called amnestic dyskinesia, to include those two patients.

6.4 Control recruitment and sampling:

Controls had lumbar puncture performed as a part of their neurological evaluation. The aim was to include a group of age-matched controls.
In Sønderborg:
As the physician performing the lumbar puncture due to my affiliation with that department, I was able to contact these controls by phone prior to their appearance in the hospital setting. I was able to inform them orally about the project and to ask them to be fasting prior to the lumbar puncture. At the day of the lumbar puncture we again talked about the project and written/oral consent was given. The lumbar puncture and blood sampling procedure as well as the following samples processing procedure did not differ from patients.
Permission was given to go through the patient's medical history, including medicine use, hereditary diseases, previous illnesses etc.
In Odense:
Due to a need for more controls for the study, we gained permission from the ethical committee in the Region of Southern Denmark to recruit controls from the Dept. of Neurology in Odense, identifying patients who had lumbar puncture performed as a part of their check-up. For this group, due to ethical reasons, I was not able to contact these patients on beforehand; thus I could not ask them to be fasting. Patients were informed about the project when they came for the general information about the lumbar puncture procedure. They were

given both oral and written information, and oral/written consent was given prior to the inclusion in this study. For this group of controls, the lumbar puncture procedures specific to the Dept. of Neurology in Odense were followed. All subjects had the lumbar puncture done lying on the left side. Prior to sampling of CSF the pressure of the CSF (mm H_2O) was measured using a flexible spinal manometer tube attached to the spinal needle. After noting the CSF pressure CSF was drawn through the manometer tube. From this point the procedure did not differ from the previously described procedures.
For all controls in this study permission was granted to inspect the patient file for the conclusion of the neurological investigation necessitating the lumbar puncture.

6.5 Laboratory analyses:

Prior to all analyses of plasma and CSF, sample tubes were thawed slowly in ice water.

6.5.1 High-Performance Liquid Chromatography (HPLC):

HPLC analyses were performed in Associate Professor Jan Bert Gramsbergens laboratory at the Institute for Molecular Medicine, Neurobiological Research, University of Southern Denmark.
For the analysis of monoamines and metabolites HPLC is a powerful analysis tool. In HPLC a mobile liquid phase and solvent compounds of interest are forced through a column under high pressure. The column is filled with a stationary phase of low polarity (such as octadecyl carbon chain (C18)-bonded silicon dioxide); also called reversed phase HPLC. By using a polar solvent (methanol) polar compounds of interest will spend less time in the column than less polar compounds. This makes HPLC a very effective tool at separating compounds of interest. Here we use the difference in polarity between monoamines to separate them. To detect the compounds at the different time point escaping the column, we use an electrochemical detector. In this detector an electric potential is applied between two electrodes. After escaping the column, the solvent is driven in between the electrodes, and electroactive compounds (in this study, monoamines) are either reduced or oxidized at the electrode. The resulting current change is recorded as a peak on a chromatogram (mV; y-axis). By first using

a standard mix of monoamines, we are able to identify the column retention time for each monoamine, representing a peak on a specific point on the x-axis (time in minutes). The area under the peak represents the amount of a compound that has passed the electrochemical detector, and by injecting a known amount of the standard, we know the amount of a compound representing a certain area under the peak. In this way we can quantify the amount of monoamines in a sample. The electrochemical detector is extremely sensitive, making it possible to detect absolute amounts in the lower femtomol range. A more detailed description can be seen in appendix II (Andersen et al. 2017).

6.5.2 Liquid-chromatography mass-spectrometry (LC-MS):

LC-MS analyses of tryptophan (TRP) metabolites in CSF and plasma were performed by post.doc. Jesper Havelund in the laboratory of Nils J. Færgeman, Department of Biochemistry and Molecular Biology, University of Southern Denmark. In LC-MS the separation of compounds of interest uses the same column based technique as HPLC. Instead of an electrochemical detector, a mass spectrometer is used which is good for separating atoms, isotopes and fragments of molecules according to mass. The following is a very basic description of the process, and details can be read in our article (Havelund et al. 2017)(appendix III). Firstly the liquid sample from the LC-column is ionized by electrospray ionization (ESI), which is effective when dealing with liquid samples. The ionized particles are then accelerated through a negative electrical field. A magnet then deflects the ions, with heavier ions being less deflected than lighter ions. These deflected ions then hit a detector which both registers the deflection determined by where the ions specifically hit the detector plate and the abundance of ions hitting the detector plate. This creates a graph with the x-axis depicting the atomic weight of the ion (according to the deflection), and the y-axis showing the abundance of ions hitting the detector plate in that specific spot.

6.5.3 Enzyme-linked immunosorbent essay (ELISA):

The ELISA was developed separately by two research teams in the late 1960'es and gradually identified as a powerful tool for identifying specific proteins, highly applicable for clinical

medicine (Lequin 2005). Both ELISA assays were performed in the laboratory of Dr. Niels H.H. Heegard (Statens Seruminstitut). The basic procedure is as follows:
Patient samples are aliquoted into the wells of an ELISA plate. In the bottom of the wells, the capture antibody with affinity to the protein of interest (α-syn) is fixed. After incubating, the wells are washed, removing excess unbound protein. Then, a primary enzyme-linked antibody with affinity to α-syn is applied in each well, effectively pinching the protein of interest between two antibodies. After incubation and a washing procedure, rinsing off excess unbound primary antibody, an enzyme substrate is added (for both our assays streptavidin was used). The antibody-linked enzyme acts upon the substrate, and the amount of product made changes the color in each well correspondingly. A plate reader, quantifying the amount of protein in each well by comparing it to a standard, measures the wavelength of the colour of each well. The α-syn ELISA in CSF was done according to the Alpha-Synuclein ELISA Kit Protocol (Biolegend, Catalog no. 844101). The α-syn ELISA in erythrocytes is described in detail in appendix VIII. For CSF ELISA a VERSAmax microplate reader was used, for erythrocyte ELISA, a TECAN ELISAreader was used.

6.5.4 *Multiplex (Vascular Endothelial Growth Factor,* extracellular signal-regulated protein kinase 1/2*):*

Multiplex analyses were performed in the Institute of Molecular Medicine, Neurobiological Research, University of Southern Denmark. To a certain extent multiplex analysis is similar to the ELISA, also using antibodies against a substance of interest. The advantage of multiplex analysis, apart from being quicker, comes from the fact that each well in a multiplex plate contains separate areas with different capturing antibodies, permitting the simultaneous analysis of several substances. Also, this assay uses electrochemiluminescence, giving a higher sensitivity, broader dynamic range and low background noise.
The method briefly described: The mesoscale plates used in these assays contain 96 wells. In the bottom of each well, capturing antibodies are attached to a high binding carbon electrode. Samples and standards are aliquoted in the wells. After a shorter incubation time the wells are washed to remove any excess sample material that hasn't attached to the capturing antibodies. A secondary antibody with affinity to the substrate of interest is then added; since multiple substrates are evaluated simultaneously, the secondary antibody is actually a mix of

antibodies against each of the substrates of interest. The secondary antibodies are labelled with a SULFO-TAG™ label. After incubation and a washing procedure the plate is read in the plate reader. By applying a current to the well electrode, an electrochemiluminescent reaction occurs in the SULFO-TAG™ labels, emitting light, which is measured by the plate reader. Light intensity depends on the amount of secondary antibody attached, and the concentration of substrates in each well is found by comparing light intensity with the standard wells.
For both VEGF (Human Growth Factor Panel I Assay), t-ERK 1/2 and p-ERK 1/2 (Phospho(Thr202/Tyr204; Thr185/Tyr187)/Total ERK1/2 Assay Whole Cell Lysate Kit), kits from Meso Scale Diagnostics® (Rockville, Maryland, USA) were used and read on a MESO Quickplex SQ120 (Meso Scale Diagnostics, Rockville, Maryland, USA).

Deviations from manufacturer's protocol:

VEGF:
In line with the method used by Janelidze et al (Janelidze et al. 2015), samples were incubated at 4°C overnight. Otherwise the protocol followed the manufacturers instructions.
T-ERK, p-ERK:
For this assay we used CSF tubes with added phosphatase and protease inhibitors as described previously. A test assay had shown very low concentrations of these enzymes in the CSF. To increase concentrations, samples were vacuum evaporated for 24 hours before performing the multiplex assay. The dry samples were then dissolved in 25 µL of destilled water (Milli-Q, Merck KGaA, Darmstadt, Germany), quickly mixed on a vortex mixer, and the protocol of the manufacturer was followed. The percentage of p-ERK compared to t-ERK (p-ERK%) was calculated and used for analysis.

6.5.5 L-Tryptophanol (Trol) fluorescence assay:

This assay protocol was developed by Glynn Jones (PhD, Aberdeen University) and performed at the laboratory of professor Bettina Platt (Aberdeen University, The Institute of Medical Sciences) used for both CSF and plasma samples. A detailed description can be seen in the L-Tryptophanol manuscript (appendix IV). It has previously been shown that some indole-containing compounds are intrinsically fluorescent, and that the presence of oligomers (but

not fibrils) quenches the fluorescence with about 15% (Reinke *et al.* 2009). The Trol (Sigma, UK) molecule was found to be a good probe for quantifying Aβ-prefibrils, not being specific for Aβ-prefibrils but also other prefibrillary amyloids(Reinke *et al.* 2010). It is to be noted though, that Trol does not seem to be quenched by prefibrillary α-syn (Reinke et al. 2010).

6.5.6 Glucocerebrosidase enzyme activity fluorescence assay:

The GCase assay was performed in the laboratory of associate professor Jan Bert Gramsbergen, Institute of Molecular Medicine, Neurobiology Research, University of Southern Denmark. Briefly, this assay measures GCase activity by the conversion of the GCase substrate 4-Methylumbelliferyl beta-D-glucopyranoside(MUGIc)(Sigma, M3633) to the product 4-Methylumbelliferone. CSF samples and MUGIc are mixed in the wells of a fluorescent plate and incubated for 24 hours. Standard wells are made with different known concentrations of the product 4-Methylumbelliferone. Adding a stop-solution then stops the enzymatic activity, and the plates are read in a fluorescent plate reader. The higher the GCase activity in a given sample, the more substrate will be converted into the product. This is measured as a change in fluorescence in the plate reader. The amount of product is then calculated by plotting well values in a standard curve. GCase activity (mU/mL) is then calculated with one U being defined as the amount of enzyme that hydrolyzes 1 nmol of substrate pr. minute at 37°C
Dividing individual values with the control mean created normalized values used for analysis. For a detailed description please see appendix IX.

6.5.7 Western blot assay (α-synuclein subspecies):

Western blot (WB) assays were performed by Peter Lønsmann Iversen (Cand.Scient.) and Tomasz Brudek (Cand.Scient, PhD)(Research Laboratory for Stereology & Neuroscience, Bispebjerg, Copenhagen University Hospital).
A detailed description of the method is described in the master thesis by Peter Lønsmann Iversen (2017, University of Copenhagen, Department of Biology).

The WB method uses electrophoresis to separate proteins according to different features such as molecular weight or charge. For these analyses, proteins are separated by molecular weight.

Initially, proteins in CSF samples are denatured, which results in the loss of their secondary and tertiary structure. This way, proteins can be selectively separated by size, measured in kilo-Dalton (kDa). The proteins in the CSF samples are then covered with sodium-dodecyl sulfate (SDS), effectively applying a negative charge to the proteins. Samples are then transferred into wells in one end of a polyacrylamide gel (PAG). By applying a current with the anode in the opposite end of the PAG, the now negatively charged protein particles are drawn through the PAG through electrophoresis (E). The entire procedure is therefore called SDS-PAGE. Due to the size of the channels in the PAG, smaller proteins move faster through the PAG, effectively separating proteins by size.

The PAG is then sandwiched between a blotting pad and a nitrocellulose membrane, and the proteins in the PAG are transferred onto the blotting pad by applying a current perpendicular to surface of the PAG. A primary antibody is then incubated on the blotting pad. The antibodies are aimed at specific epitopes of the α-syn protein (see figure 10). I will shortly mention the primary antibodies used: Antibodies HLU-N1 (Lundbeck A/S), 5-G4 (Millipore, USA; CN MABN389), MJFR1 (Abcam, USA; CN ab138501), syn-221 (Santa Cruz Biotechnology, USA; CN sc-12767) and HLU-C4 (Lundbeck A/S) identify normal epitopes on the α-syn protein. Ser-87 (Santa Cruz Biotechnology, USA; CN sc-19893-R) specifically binds to α-syn phosphorylated at the serine 85 residue, Tyr123-/133 (Novus Biologicals, USA; CN NBP1-26380) binds to α-syn nitrosylated at tyrosine residue 125 or 133, and ser-129 (Santa Cruz Biotechnology, USA; CN sc-135638) binds to α-syn phosphorylated at serine residue 129. Subsequently, a secondary antibody with a fluorescent label, binding to the primary antibody, is added. Quantification of the protein of interest is then done using a near-infrared imaging system.

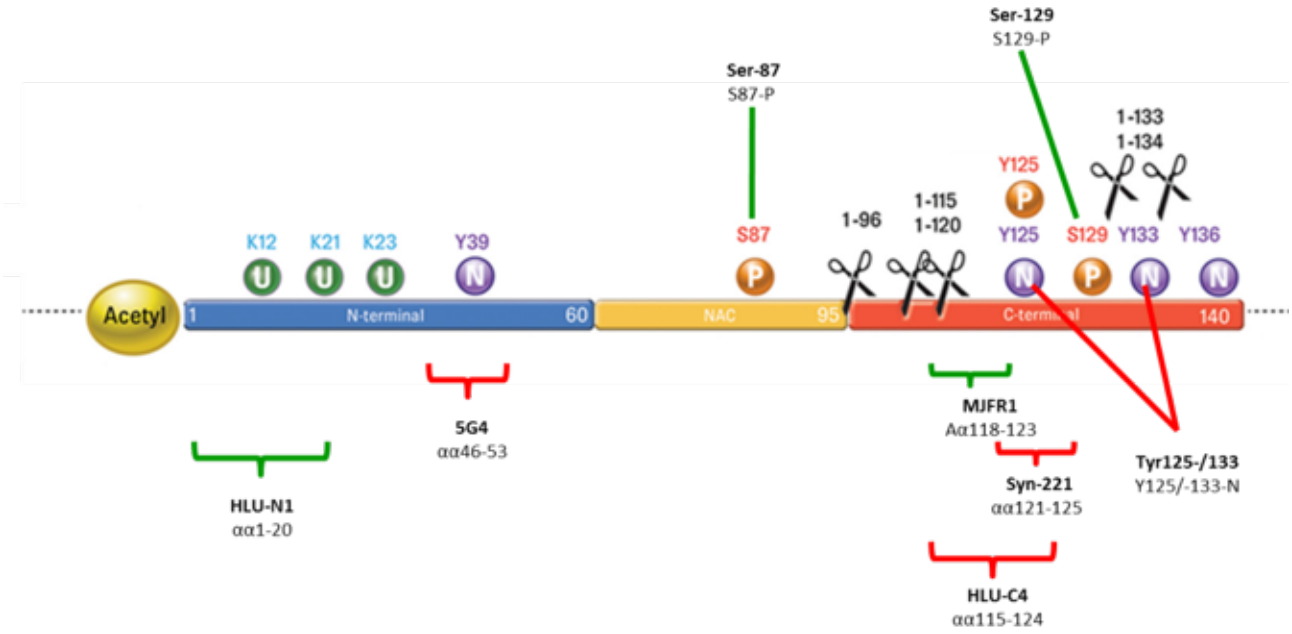

Figure 9: An epitope map of the primary antibody binding site on the α-syn protein. Potential posttranslational modifications of α-syn are shown: Green circles indicate ubiquitination, purple circles indicate nitrosylation, orange circles indicate phosphorylation, and scissors indicate truncation. Figure as presented in master thesis of Peter Lønsmann Iversen (2017), adapted from (Schmid et al. 2013).

6.6 Statistical analysis:

Statistical analysis was performed using STATA…. Continuous data was tested for Gaussian distribution visually by histogram as well as a qq-plot. Shapiro-Wilks test for normality was additionally used. Parametric data was analysed using Student's t-test between two groups. Non-parametric data was analysed using the Wilcoxon rank-sum test between two groups. Analysis of variance between the four groups in this study was done by ANOVA for parametric data and Kruskall-Wallis for non-parametric data. For parametric data, pairwise comparison was performed subsequently using Bonferronis multiple comparisons. Dunn-test was used for multiple pairwise comparisons for non-parametric data; only for the catecholamine analyses was Bonferronis correction for multiple comparisons not performed. Receiver operating characteristics (ROC) was performed using a non-parametric ROC analysis. The maximal Youden's index score was determined by the formula (sensitivity+specificity-1). With binary variables as the dependent variable, a logistic regression analysis was used. With continuous

variables linear regression analysis was used. All linear regression analyses were performed using bootstrapping with 1000 repetitions.

All analyses have been made both including and excluding the DLB and AD patients.

In the graphical presentation of results, error bars represent mean ± standard error of mean.

Statistical significance is presented as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

CHAPTER 7: RESULTS

7.1 Patient and control data:

As can be seen in table 1, all patients fulfil the UKBBC, suffering form bradykinesia, 88% showing various degrees of resting tremor, 85% suffering from rigidity. 15 of the 26 patients had a DAT-scan performed as part of their diagnostic work up, showing either bilateral or unilaterally decreased uptake of FP-CIT in the basal ganglia. 92% had a unilateral debut, with 81% continuing to show an asymmetrical symptomatology with the debut side most affected.
One patient fulfilled the inclusion criteria for PD and showed marked bilateral decrease of FP-CIT-uptake, but developed severe cognitive deficits in an early disease stage. The patient was later diagnosed as suffering from DLB, but was included in the analyses, which will be discussed later.
In table 2, more clinical data for each patient is described. Age ranged from 41-76 years, with 8 patients not receiving L-DOPA (PD-N), 8 receiving L-DOPA but not dyskinetic (PD-L), 10 L-DOPA treated and dyskinetic (PD-LID). Symptom duration ranged from 1.5 to 17 years, significantly correlated with the use of L-DOPA (Linear regression analysis, $p=0.002$). The UPDRS part III motor score ranged from 8-53, and UDysRS objective scores ranged from 0-15 in the dyskinetic patient group. The use of Amantadine has not been registered in table 2; only three of the dyskinetic patients were treated with Amantadine.
Cognitive assessment using the MOCA score showed that patients treated with L-DOPA had significantly lower scores than PD-N ($p=0.031$). Although L-DOPA treated patients were not significantly older than PD-N, MOCA scores are negatively correlated to age ($p=0.001$, $R^2=0.24$).
Patient and control data are compared in table 3. Controls are significantly younger than both the total PD group and PD-L, with a preponderance of men in the total PD group compared to controls. CSF protein concentrations are significantly higher in the total PD-group and in the PD-N group compared to controls.

atient	criterium												
	Bradykinesia	Resting tremor	Rigidity	Postural instability	Unilateral onset	Resting tremor	Progression	Debute side most affected	Excel-lent L-DOPA response	L-DOPA-induced dyskine-sia	L-DOPA response > 5 years	Clinical course >10 years	Decreased FP-SPECT uptake
	✔	✔	✔		✔	✔	✔	✔		✔	✔	✔	✔, unilateral
	✔	✔			✔	✔	✔	✔	✔				
	✔	✔	✔	✔		✔							✔, bilateral
	✔		✔		✔		✔	✔					
	✔	✔			✔	✔	✔	✔	✔			✔	
	✔	✔	✔		✔	✔	✔						
	✔	✔	✔	✔	✔	✔	✔	✔	✔				
	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
	✔	✔	✔		✔	✔	✔	✔	✔	✔			✔, unilateral
	✔	✔	✔		✔	✔	✔	✔	✔	✔			✔, unilateral
	✔	✔	✔		✔	✔	✔	✔					✔, bilateral
	✔	✔	✔		✔	✔	✔	✔		✔			✔, unilateral
	✔	✔	✔		✔	✔	✔			✔			✔, bilateral
	✔	✔	✔	✔	✔	✔	✔	✔					✔, bilateral
	✔	✔	✔	✔	✔	✔		✔					✔, unilateral
	✔	✔	✔	✔	✔	✔	✔	✔				✔	✔, unilateral
	✔	✔	✔	✔	✔	✔		✔					✔
	✔	✔	✔		✔	✔	✔						✔
	✔	✔	✔	✔	✔	✔	✔	✔					✔
	✔	✔	✔	✔	✔	✔	✔	✔	✔				
	✔	✔		✔	✔		✔	✔		✔			✔
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	✔		✔		✔		✔	✔		✔	✔	✔	
	✔	✔	✔	✔	✔	✔		✔					
	✔		✔		✔		✔	✔	✔	✔		✔	✔
	✔	✔	✔	✔	✔	✔		✔					

Table 1: The United Kingdom Brain Bank criteria assessed for each PD patient.

Sex	Age (years)	Disease duration (years)	MAO-I	DA-agonist	L-DOPA	Years of L-DOPA treatment	Daily L-DOPA-equivalent dosage (mg)	UPDRS III motor score	UDysRS objective score	Amnes-tic dyskine-sia	MOCA score	MMSE score	CSF available	Blood sample available
Male	46	17	Yes	Yes	Yes	8	862,5	38	N/A	1	N/A	29	✔	✔
Male	73	10	No	Yes	Yes	8	600	35	N/A	0	N/A	28	✔	✔
Male	76	1,5	No	No	Yes	1	300	29	N/A	0	N/A	19	✔	✔
Male	66	3	Yes	Yes	No	0	300	17	0	0	28	30	✔	✔
Male	75		No	Yes	Yes	N/A	510	34	0	0	23	30	✔	✔
Male	66	5	Yes	Yes	No	0	310	29	0	0	29	29	✔	✔
Male	67	6	Yes	Yes	Yes	N/A	1385	53	3	1	24	30		✔
Male	63	15	No	No	Yes	8	800	16	13	1	24	29	✔	✔
Male	60	7	No	No	Yes	4	500	36	11	1	27	30	✔	✔
Male	70	2,5	Yes	Yes	No	0	410	15	0	0	28	30	✔	✔
Male	75	10	No	No	Yes	1	450	40	0	0	23	28	✔	✔
Female	47	7	No	Yes	Yes	2	510	17	0	1	28	30	✔	✔
Male	60	4	No	Yes	Yes	0	720	23	8	1	25	29	✔	✔
Female	55	2,5	Yes	Yes	No	0	380	18	0	0	26	29	✔	✔
Female	60	2	No	Yes	No	0	320	8	0	0	30	30	✔	✔
Male	41	10	Yes	Yes	Yes	0,3	730	44	0	0	29	30	✔	✔
Male	51	8	Yes	No	No	0	100	17	0	0	30	30		✔
Male	65	3,5	No	Yes	Yes	0,3	470	29	0	0	26	30	✔	✔
Male	66	1,5	No	Yes	No	0	105	18	0	0	24	26	✔	✔
Male	62	8	No	Yes	Yes	4	1643,75	32	0	0	28	30		✔
Female	57	6	Yes	Yes	Yes	2,4	1060	36	7	1	27	28	✔	✔
Male	66	6	No	Yes	Yes	1	620	33	0	1	27	28		✔
Male	68	10	No	No	Yes	7	950	17	13	1	26	29	✔	✔
Female	67	7	Yes	Yes	No	0	420	28	0	0	29	30	✔	✔
Female	65	10	No	Yes	Yes	N/A	1160	32	15	1	28	29		✔
Male	72	3,5	No	No	Yes	0,7	600	50	0	0	25	29	✔	✔

Table 2: Clinical data and data on medicine use for each PD patient. MAO-I; Monoamine-oxidase inhibitor, DA; dopamine, L-DOPA; Levodopa, UPDRS; Unified Parkinson's Disease Rating Scale, UDysRS; Unified Dyskinesia Rating Scale, MOCA; Montreal Cognitive Assessment, MMSE; Mini-Mental State Examination, CSF; Cerebrospinal fluid

Patient and control comparison

	Control (n=16)	PD-N (n=8)	PD-L (n=8)	PD-LID (n=10)	PD total (n=26)
Age	55.1 ± 11	62.6 ± 6.6	67.4 ± 11.8*	59.9 ± 7.9	63 ± 9.1*
CSF protein (g/L)	0.34 ± 0.08	0.5 ± 0.18**	0.44 ± 0.14	0.45 ± 0.15	0.46 ± 0.15**
Gender (m/f)	8/8	5/3	8/0	7/3	20/6
Disease duration (years)		3.9 ± 2.4§	6.6 ± 3.7	8.8 ± 4.2	6.6 ± 4.0
UPDRS III motor score		18.8 ± 6.8 †††, §	36.6 ± 7.5	30.1 ± 11.8	28.6 ± 11.5
UDysRS objective		0	0	7.8 ± 5.7	3.0 ± 5.2
MOCA		28 ± 2	25.7 ± 2.5	26.2 ± 1.6	26.7 ± 2.2
L-DOPA equivalent daily dose (mg) (L-DOPA only)	0	0	439 ± 329	593 ± 247	363 ± 343
L-DOPA equivalent daily dose (mg)(total)	(One patient on 8 mg Ropinirol = 160)	293 ± 126 ††, § § §	663 ± 416	857 ± 288	624 ± 374

Continuous parametric data, Student's t-test between groups; non-parametric data, Mann-Whitney test between groups
*: vs. control; * p<0.05, ** p<0.01
†: vs. PD-L; † p<0.05, †† p<0.01, ††† p<0.001
§: vs. PD-LID; § p<0.05, § § § p<0.001

Table 3: Demographic and clinical data in controls and PD groups.

7.2: Diagnostic biomarker candidates

7.2.1 Alpha-synuclein

ELISA CSF total alpha-synuclein (CSF-t-α-syn)(Fig.10A):

No significant difference between PD total and controls were found. CSF t-α-syn was significantly correlated with age in the control group when including the two AD patients (p=0.023). Gender did not affect CSF t-α-syn. A multiple regression analysis of CSF-t-α-syn including case, age, and gender did not show significant correlations.

In the PD group CSF-t-α-syn was not correlated to L-DOPA-LED, total LED, the UPDRS part III motor score or MOCA. Significant correlation with MMSE-score was due to the DLB patient having a marked decrease in score, and no significance was attained without the DLB patient.

ELISA erythrocyte total alpha-synuclein (ery-t-α-syn)(Fig.10B):

No significant difference between PD total and controls were found. Neither age nor gender affected ery-t-α-syn levels. No correlation was found between ery-t-α-syn and CSF-t-α-syn, disease duration, L-DOPA-LED, total LED, MOCA scores, MMSE scores, or UPDRS part III motor scores.

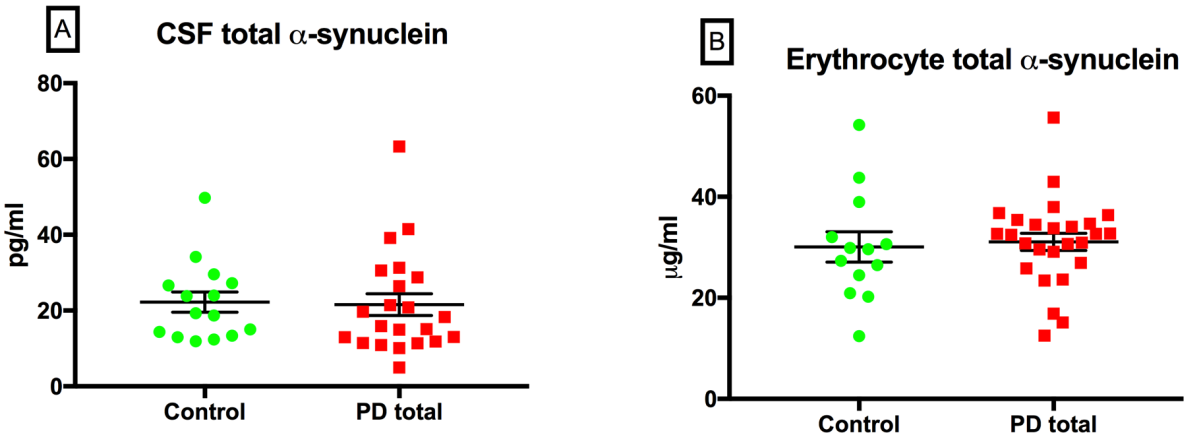

Fig.10: ELISA analysis of CSF (A) and erythrocytes (B). (A) Student's t-test, not significant (B) Student's t-test, not significant.

Western blot

Results from using the antibodies MJFR1, syn-211 and ser-129 were not significant and are not shown.

A significant decrease in PD CSF α-syn levels was found when focusing on full length α-syn monomers as well as one α-syn dimer. As seen in figure 10, using antibodies against 5-g4 (Fig.11A), ser-87 (Fig.11B), tyr-125/133 (Fig.11C, only significant when including the AD and DLB-patients), HLU-C4 (Fig.11D), and HLU-N1 (Fig.11E, only significant when excluding AD and DLB-patients) revealed differences between PD and controls. Controls had significantly higher levels of dimeric α-syn visualized with the HLU-C4 antibody (Fig.10F, only when including AD and DLB patients) Age and gender had no effect on α-syn levels. Using the 5-G4 and ser-87 antibodies (Fig.12B,C), the tetrameric/monomeric α-syn ratios were significantly higher in PD patients (5-G4: $p=0.0023$, ser-87: $p=0.0013$). Age and gender had no effect on these ratios. In controls dimer HLUC4 levels significantly correlated with age ($p=0.03$, $R^2=0.4$). A negative correlation between MOCA scores and full length α-syn monomer levels, specifically 5-g4 (Fig.12A).

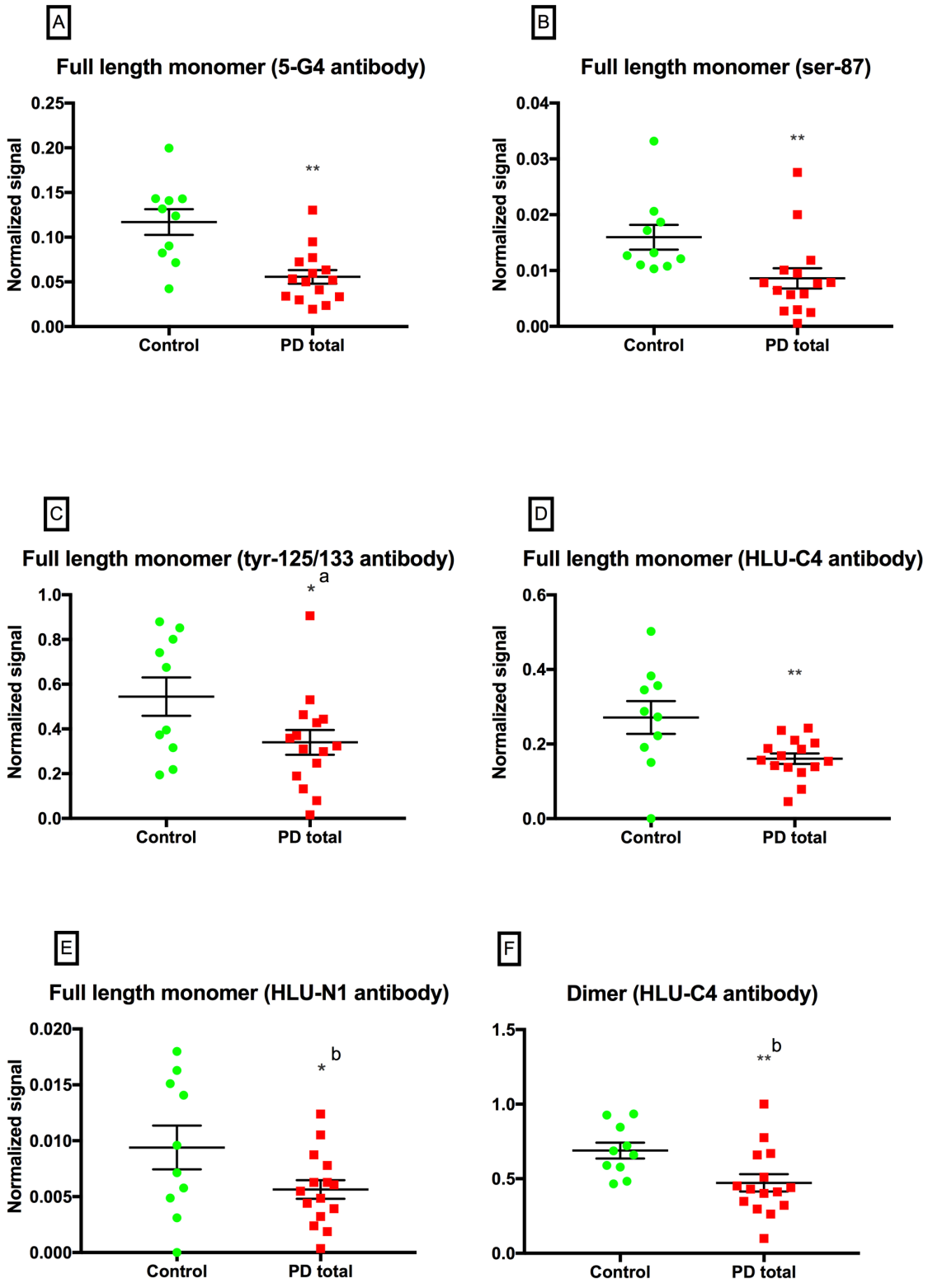

Figure 11: Western blot analysis. (A): Wilcoxon rank-sum test, p=0.0016 (B) Wilcoxon rank-sum test, p=0.0023 (C) Student's t-test, p=0.0462 (D) Student's t-test, p=0.0205 (E) Student's t-test, p=0.0497 (F) Student's t-test, p=0.0164.

a: Only significant when including DLB and AD patients

b: Only significant when excluding DLB and AD patients

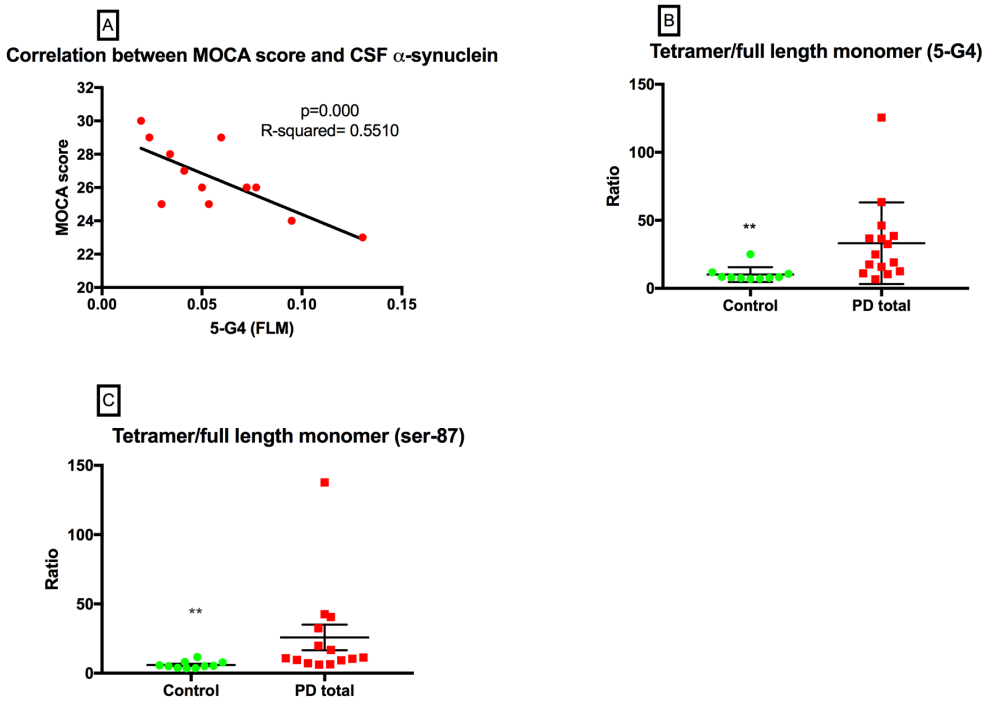

Figure 12: (A) Correlation between MOCA scores and full length monomeric α-syn visualized using the 5-G4 antibody. Linear regression with bootstrap and 1000 repetitions. (B) & (C) show the ratio between tetramers and full length monomers the 5-G4 and ser-87 antibody respectively. (B) Wilcoxon rank-sum test, p=0.0023 (C) Wilcoxon rank-sum test, p=0.0013. FLM; Full length monomer.

7.2.2 L-Tryptophanol assay

CSF TROL:

Trol scores in PD CSF are significantly lower compared to controls, also if excluding DLB patient from the PD group (Fig.13A). Age is significantly correlated to CSF Trol only in PD CSF when excluding the DLB patient. Gender does not affect CSF Trol scores. Using multiple regression analysis including case and age shows that both PD status and age affect CSF Trol scores, although PD status had a more marked effect.

In the PD group, disease duration is negatively correlated to the CSF Trol score (Fig.15). CSF Trol score is not correlated to L-DOPA-LED, total LED, MOCA- or MMSE-scores, or UPDRS part III motor scores.

Plasma Trol:

PD patients had significantly higher plasma Trol scores than controls, specifically L-DOPA treated patients (Fig.13B). Age was significantly correlated to plasma Trol scores in total

(p=0.003), and also specifically in the PD group (p=0.018), but not in controls specifically. Females had significantly lower plasma Trol scores in total (p=0.016), as well as in the PD group when excluding the DLB patient (p=0.049). In a multiple regression analysis including case, age and gender, only age was significantly correlated to plasma Trol scores.
In the PD group, plasma Trol scores were not correlated to disease duration, L-DOPA-LED, total LED, MOCA- or MMSE-scores, or the UPDRS part III motor score.

Plasma/CSF Trol ratio:
In PD we found a significantly higher plasma/CSF Trol ratio than controls, specifically in L-DOPA treated patients (Fig.13C). Age was not significantly correlated to the plasma/CSF Trol ratio. In total females had significantly lower ratios, but not in PD and control groups separately.
Multiple regression analysis including case, age and gender showed that PD status significantly increased the plasma/CSF Trol ratio whereas being female to a lesser extent was negatively correlated to the plasma/CSF Trol ratio.
In the PD group the plasma/CSF Trol ratio was not correlated to disease duration, L-DOPA-LED, total LED, MOCA- or MMSE scores or the UPDRS part III motor score.

CSF Trol/CSF protein ratio:
Both PD total and all three PD groups had significantly lower CSF Trol/CSF protein ratios than controls (Fig.13D). Age did not affect the ratio, whereas females had significantly higher ratios in total. This was not found in the PD or control group separately. Performing multiple linear regression analysis including case, age, and gender, PD status was markedly correlated with a decreased CSF Trol/CSF protein ratio.
Non-parametric ROC analysis, excluding DLB and AD patients from analysis, yielded an AUC of 0.8773. At the best cut-off found by Youden's index, calculating the CSF Trol/CSF protein ratio had a 90% sensitivity, 73% specificity, and a positive predictive value of 83% (Fig.14).
In the PD group the ratio was negatively correlated with disease duration, when excluding the DLB patient. No correlation was found between the ratio and L-DOPA-LED, total LED, MOCA- or MMSE-scores, or the UPDRS part III motor score.

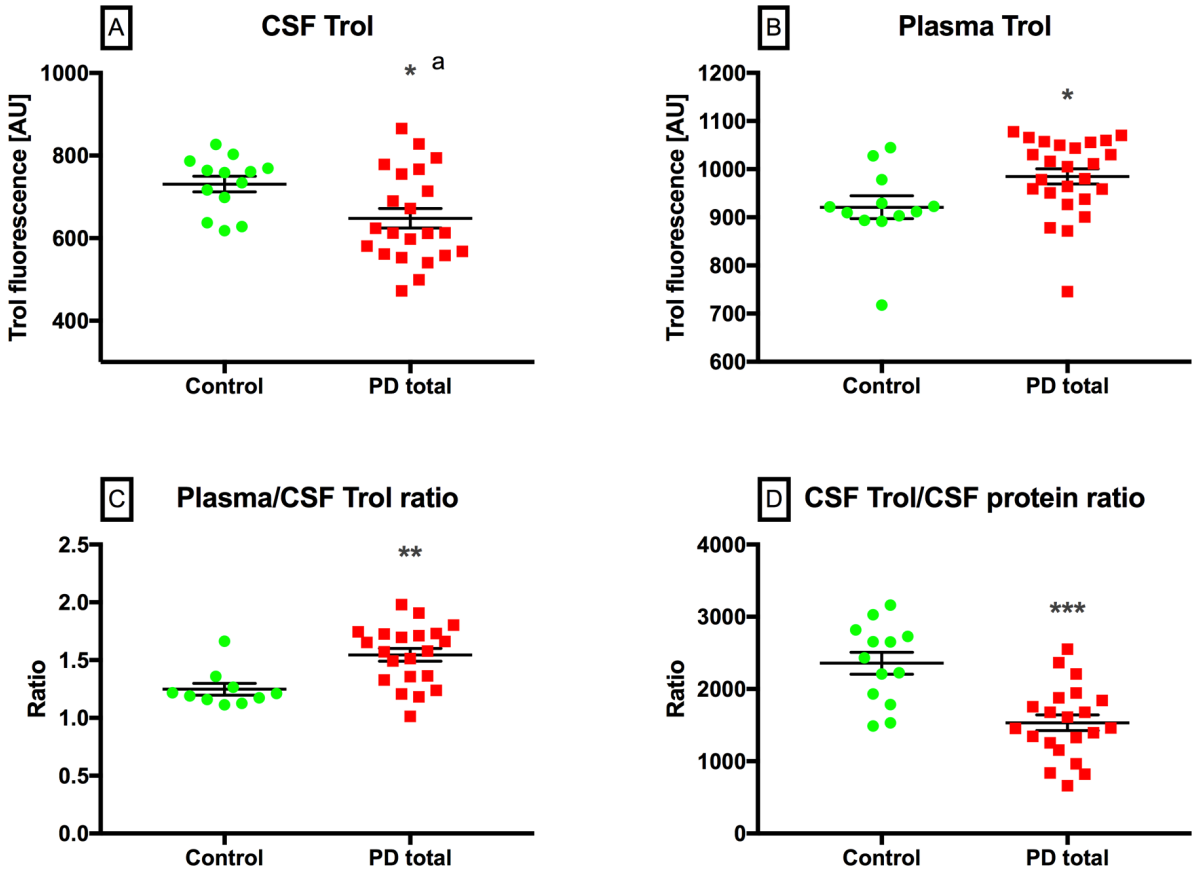

Figure 13: L-Tryptophanol assay comparing PD total and controls. (A) Student's t-test, p=0.0203 (B) Wilcoxon rank-sum test, p=0.0137 (C) Student's t-test, p=0.0022 (D) Student's t-test, p=0.0001.
a: Not significant when excluding both AD and DLB patients, still significant when only excluding DLB patient.

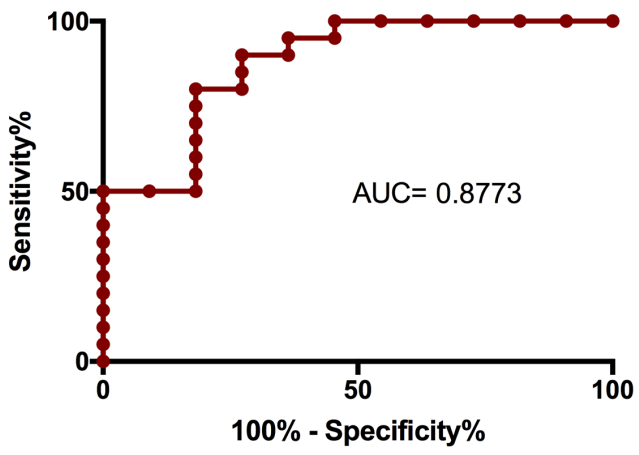

Figure 14: Receiver operating characteristics for CSF Trol/CSF protein-ratios. AUC; Area under the curve.

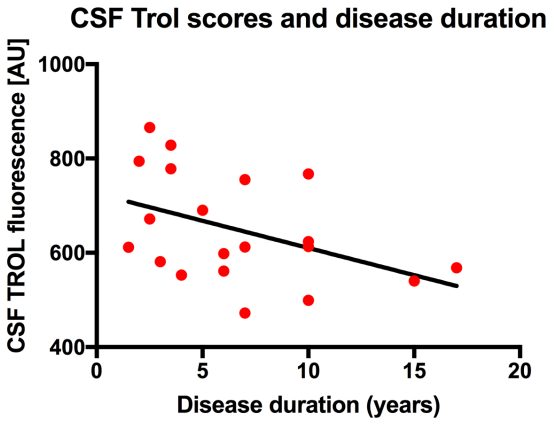

Figure 15: Correlation between CSF Trol scores and disease duration. Linear regression analysis with bootstrapping and 1000 repetitions.

7.2.3 Glucocerebrosidase

PD patients had significantly lower GBA activity than controls (Fig.16A). Age was negatively correlated to GBA activity in the total group as well as in the control group (only when including AD patients). Gender did not affect GBA activity. Using multiple regression analysis correcting for age, we still found significantly lower GBA activity in PD CSF. Excluding AD patients and the DLB patient, age was no longer significantly correlated to GBA activity, whereas patient status was.

GBA activity was significantly correlated with the total LED. No correlation was found between GBA activity and disease duration, L-DOPA-LED, MOCA- and MMSE-scores, or UPDRS part III motor scores. Correcting for the total protein content in CSF by dividing normalized GCase activity with CSF total protein as suggested by another study(van Dijk *et al.* 2013), results were even more significant (Fig.16B). ANOVA showed significant differences between groups (p=0.0000), and subsequent Bonferroni multiple comparison showed significantly lower ratios of normalized GCase activity compared to protein concentration in all PD groups compared to controls. Excluding the DLB and AD patients, ROC analysis yielded an AUC of 0.9462, with the best cutoff at <1.838, showing a sensitivity of 75%, specificity of 100% and positive predictive value of 94%. The GCase corrected/CSF total protein variable in PD and controls was significantly correlated to age (p=0.007) and female subjects had lower values (p=0.011), but multiple linear regression showed no significant correlation to age or gender when comparing PD to controls.

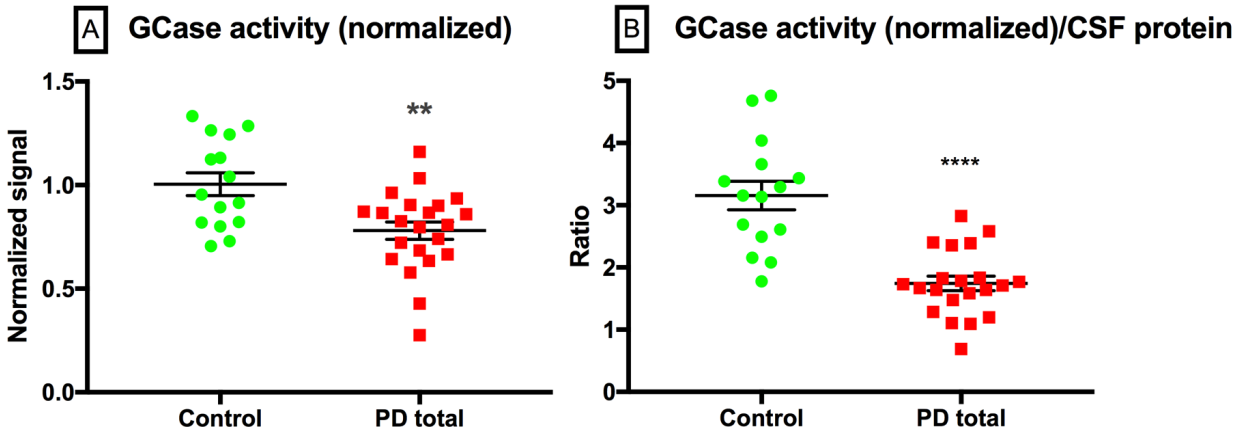

Figure 16: GCase activity in CSF in PD and controls. (A): Student's t-test, p=0.0024 (B): Student's t-test, p=0.0000.

7.2.4 Vascular Endothelial Growth Factor:

CSF VEGF levels were significantly lower in control CSF compared to PD CSF (Fig.17), especially between controls and PD-L. The PD-L group was significantly older than the control group, and age was markedly correlated with VEGF levels in PD+controls and in the control group. In the control group females had significantly lower VEGF levels, even though they were not significantly younger than males. In multiple regression analysis taking into account age and gender, age was the only significant factor.
In the PD group there was no correlation between VEGF and disease duration, L-DOPA-LED, total LED, MOCA- and MMSE-scores, or UPDRS part III motor scores.

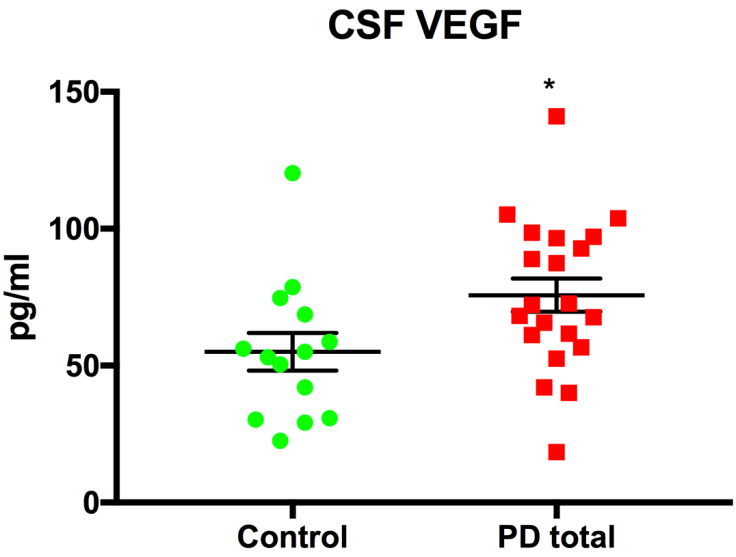

Figure 17: Vascular endothelial growth factor in CSF. Student's t-test, p=0.0321.

7.2.5 Catecholamines:

As a diagnostic biomarker focus was put on patients not receiving L-DOPA treatment, which significantly alters the monoamine profile.

As can be seen in figure 18, patients not receiving L-DOPA treatment had significantly lower levels of L-DOPA (Fig.18A), DA (Fig.18B), DOPAC (Fig.18C) and HVA (Fig.18D). In the control group, non-fasting subjects had significantly higher DOPAC levels than fasting subjects.

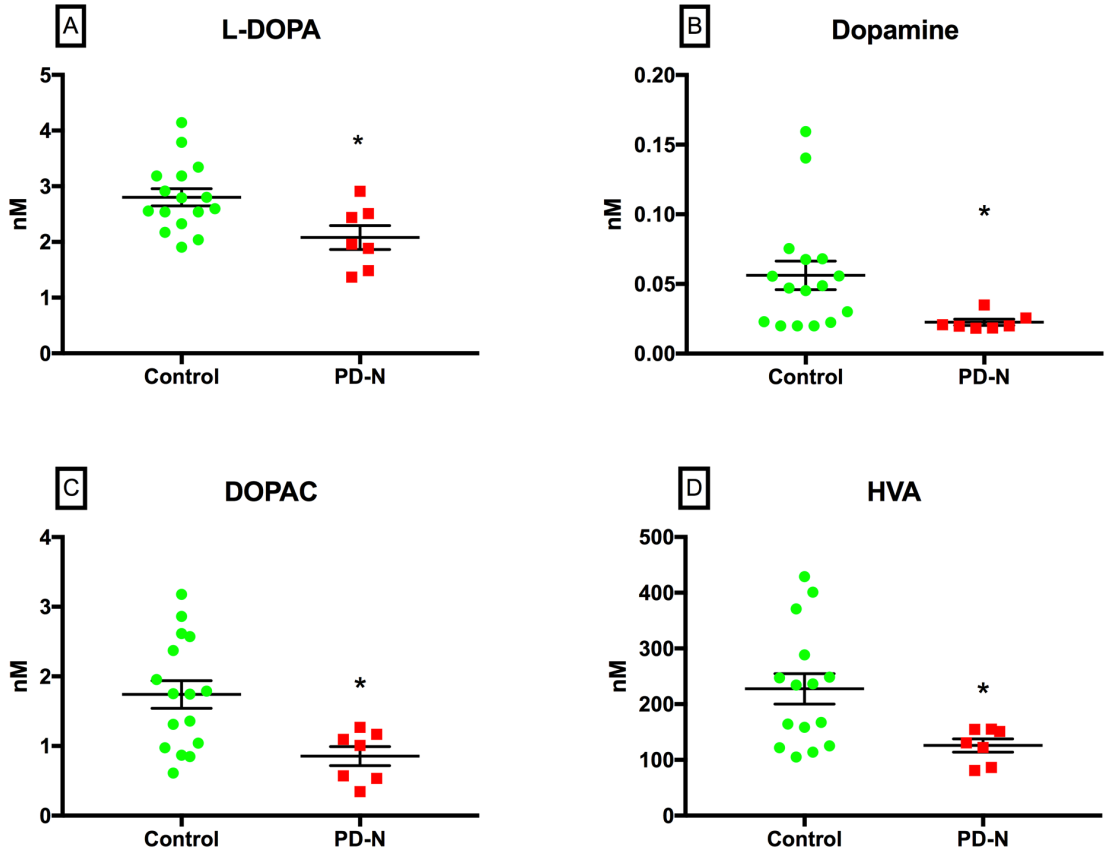

Figure 18: Catecholamine metabolites in CSF in controls and PD patients not treated with L-DOPA. (A) Student's t-test, p=0.0146 (B) Wilcoxon rank-sum, p=0.0067 (C) Student's t-test, p=0.0108 (D) Student's t-test, p=0.0235.

7.2.6 Tryptophane metabolism:

Plasma:

Focusing on tryptophane (TRP) and TRP metabolites, there are no significant differences between PD and controls, aside from an increase in 3-O-methyldopa (3-OMD) (p=0.0014), 5-hydroxytryptamine (5-HT) (p=0.0059), and L-DOPA (p=0.0002). Linear regression analysis taking L-DOPA intake into account clearly showed that an increase in these variables was due to L-DOPA-intake (p=0.000 for each). Non-fasting did not significantly affect plasma levels of any of the metabolites except 3-OH-anthranilic acid (3-HANA) levels (linear regression analysis with 3-HANA as dependent variable, p=0.0156), and significantly higher levels in male compared to female controls were identified regarding anthranilic acid (ANA), TRP, xanthurenic acid (XAN) and KYN.

The 3-HK/KYNA ratio overlaps between PD and controls, but is significantly higher in PD (p=0.0107)(not depicted in a figure). This difference is driven by an increase in PD-LID (see segment 6.3.2). The 3-HANA/3-HK ratio is significantly lower in PD (p=0.0025)(not depicted in a figure), specifically in L-DOPA treated patients (see segment 6.3.2). In PD plasma ANA and the plasma 3-HANA/3-HK ratios are significantly correlated (p=0.033, R-squared=0.1655).
In the PD population no significant correlation was found between variables and the UPDRS part III motor score.
Linear regression analysis revealed correlations between the MOCA score and several variables: Correlation with TRP (p=0.006, R^2=0.21) and XAN (p=0.039, R^2=0.14). TRP and XAN were negatively correlated with age (p=0.011, p=0.003 respectively), and taking age into account only provided a near significant correlation between TRP and MOCA scores (p=0.05), but not XAN. Negative correlation with 3-OMD (p=0.007 R^2=0.18), 5-HT(p=0.000 R^2=0.25), and L-DOPA (p=0.000). As mentioned previously, patients receiving L-DOPA have significantly lower MOCA scores than PD-N. All three variables were significantly correlated to L-DOPA intake (p=0.000 for all), as well as a negative correlation between age, 3-OMD (p=0.002) and 5-HT (p=0.049), and taking these into account revealed no significant correlation with MOCA scores. KYN levels were not affected by age or L-DOPA intake, and correlated with MOCA scores (p=0.015, R^2=0.14)(Fig.19).

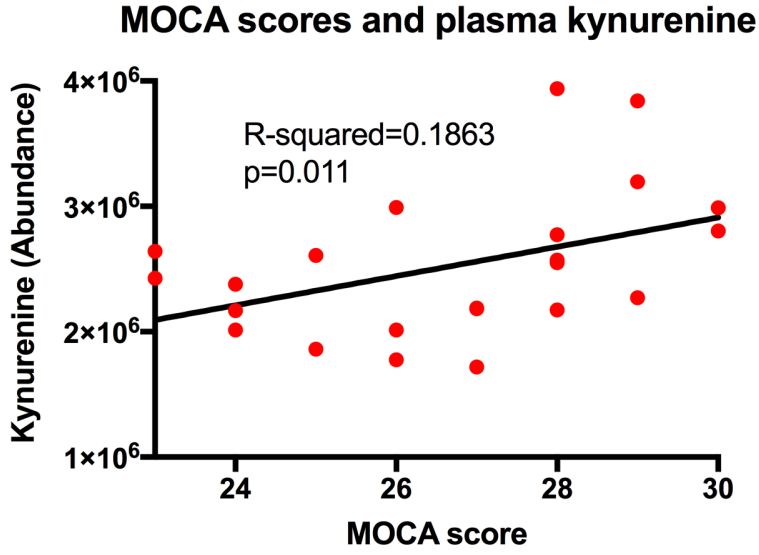

Figure 19: Correlation between plasma kynurenine levels and MOCA scores in PD patients. Linear regression analysis with bootstrapping and 1000 repetitions.

CSF:

ANA and 5-HIAA levels are increased and decreased respectively in PD (p=0.0109, p=0.0158 respectively)(Fig.20A,B). Neither ANA nor 5-HIAA levels correlated with age or fasting status, and when comparing L-DOPA treated patients with PD-N, there was no significant difference in ANA and 5-HIAA levels.

An increase in L-DOPA and 3-OMD levels in PD was due to L-DOPA intake.

In the PD group, no significant correlation between variables and UPDRS part III motor score was found.

MOCA scores correlated negatively with ANA (p=0.033, R^2=0.19)(Fig.20C), a variable not correlating with age. Omitting the DLB patient did not change the results.

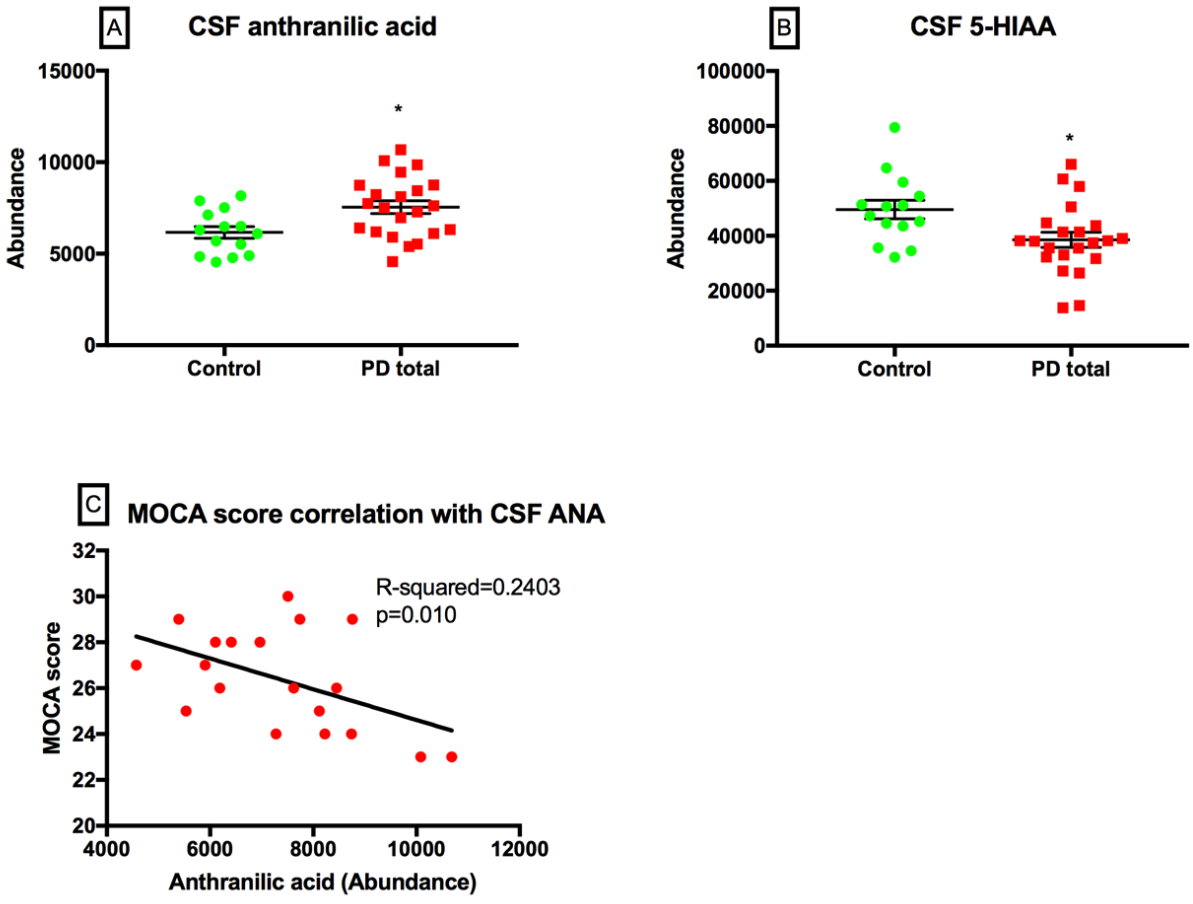

Figure 20: Differences in CSF tryptophane metabolite levels of anthranilic acid and 5-HIAA in PD versus controls and correlation between MOCA scores and CSF ANA in PD. (A) Student's t-test, p=0.0109 (B) Student's t-test, p=0.0158 (C) Linear regression analysis with bootstrapping and 1000 repetitions.

7.2.7 Combination of CSF Trol/ CSF protein ratio and corrected GCase activity/CSF protein ratio:

Individually the CSF Trol and corrected GCase activity divided by the total CSF protein concentration were highly significant in separating PD from controls.
I combined the values in a single variable by multiplication:

$$\frac{CSF\ Trol}{CSF\ total\ protein} \times \frac{corrected\ GCase\ activity}{CSF\ total\ protein} = \frac{CSF\ Trol \times corrected\ GCase\ activity}{CSF\ total\ protein^2}$$

Logistic regression with case as the dependent variable and this new variable as the independent was significant (p=0.045).
Using the variable in ROC analysis, excluding the DLB and AD patients, yielded an AUC of 95%, sensitivity 85%, specificity 90.91%, PPV 82,1% and NPV 79% with a cutoff at <4555.

7.3: Biomarker candidates for L-DOPA-induced dyskinesia

7.3.1 Catecholamine metabolism in CSF:

Significant findings regarding monoamine ratios are depicted in figure 21. All analyses were performed using Dunn's test, but without correction for multiple comparisons. It was argued that each comparison tested a different hypothesis, obviating the need for multiple comparisons. For the thesis, subsequent analyses were performed with Bonferronis adjustment for multiple comparisons to observe the potential differences.
(Fig.21A) The DA/L-DOPA ratio was found to be significantly higher in the PD-LID and control group compared to PD-L. Correcting for multiple comparisons, only controls had a significantly higher ratio than PD-L, but not when excluding the AD patients.
(Fig.21B) The DOPAC/DA ratio was significantly lower in the PD-LID group compared to PD-L, PD-N and controls, and controls had significantly higher ratios than PD-N. Adjusting for multiple comparisons, only PD-LID had a significantly lower DOPAC/DA ratio than controls and PD-N. (Fig.21C) PD-N had significantly higher HVA/DA ratio compared to PD-L and PD-LID, and controls had a significantly higher ratio than PD-L. Adjusting for multiple comparisons, only PD-LID had a lower HVA/DA ratio than PD-N and controls.

(Fig.21D) Dyskinetic patients and controls have significantly higher MHPG/NA ratios than PD-L, only when not performing Bonferronis adjustment for multiple comparisons.

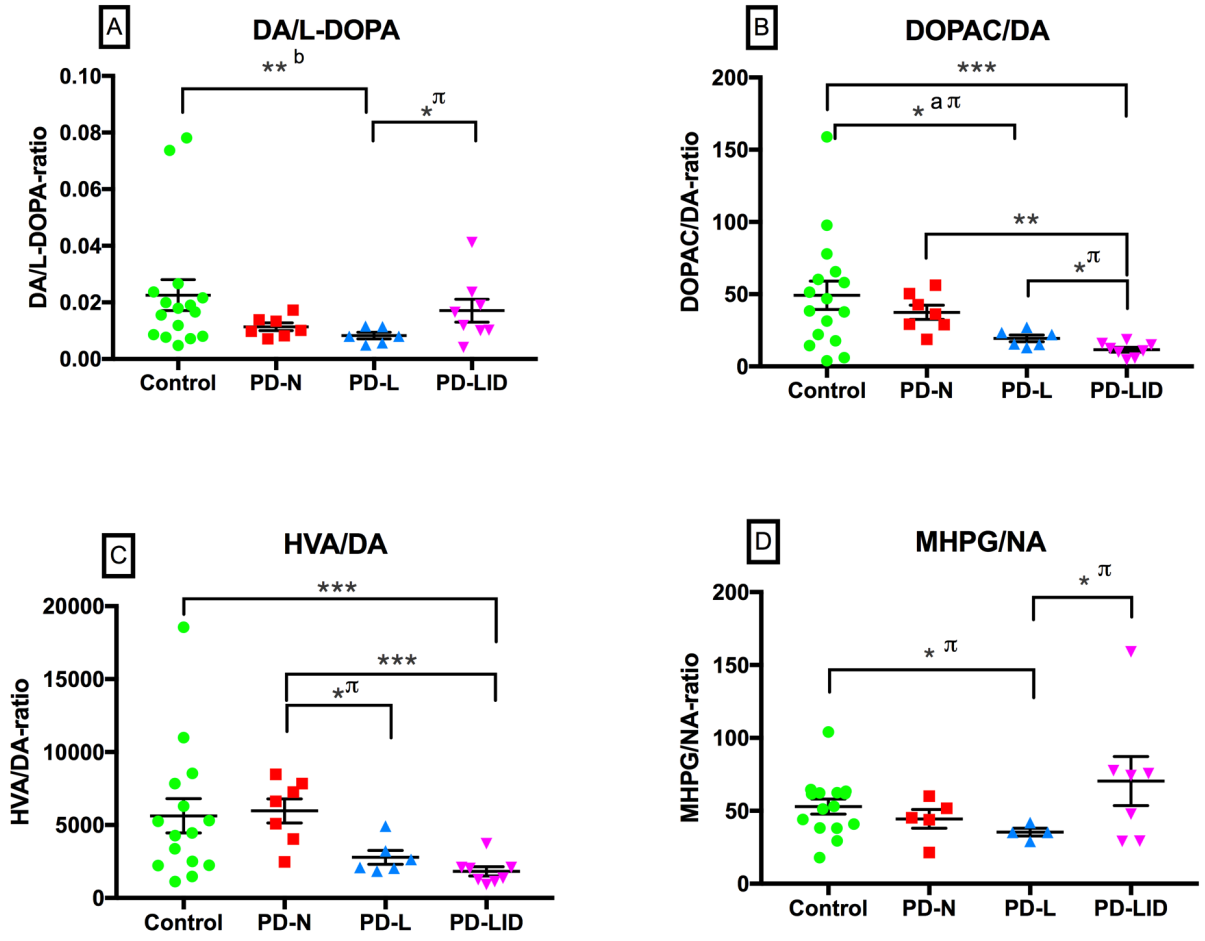

Figure 21: Catecholamine ratios in controls and PD groups. All multiple comparisons are performed using Dunn's test. (A) Kruskal-Wallis, p=0.0838 (B) Kruskal-Wallis, p=0.0018 (C) Kruskal-Wallis p=0.0019 (D) Kruskal-Wallis p=0.1410.

a: Only significant when including DLB and AD patients.

π: Not significant when using Bonferronis correction for multiple comparisons

7.3.2 Tryptophane metabolism:

Plasma:

PD-L had significantly higher ANA (only when including DLB patient)(Fig.22A) and KYNA levels than PD-LID (Fig.22B), and a significantly higher KYN/TRP ratio compared to PD-LID (Fig.22C). PD-LID had significantly higher 3-HK/KYNA (Fig.22D) and 3-HK/KYN (Fig.22E) ratios than controls and PD-N. Conversely, PD-LID had a more pronounced decrease in the 3-

HANA/3-HK ratio than PD-L (Fig.22F), as well as a lower XAN/3-HK ratio than controls and PD-N (Fig.22G).

CSF:
PD-LID (but also controls and PD-N) had significantly lower ANA levels than PD-L (p=0.0097)(Fig.23).

7.3.3 Phosphorylated Extracellular signal-regulated kinases:

Focusing on P-ERK%, ANOVA between groups was not significant. Followed by uncorrected Fisher's least significant difference (LSD) test, significant difference was found between PD-LID and controls (p=0.024)(Fig.24A). L-DOPA treated individuals had significantly higher p-ERK% than individuals not receiving L-DOPA (p=0.0318)(Fig.24B).

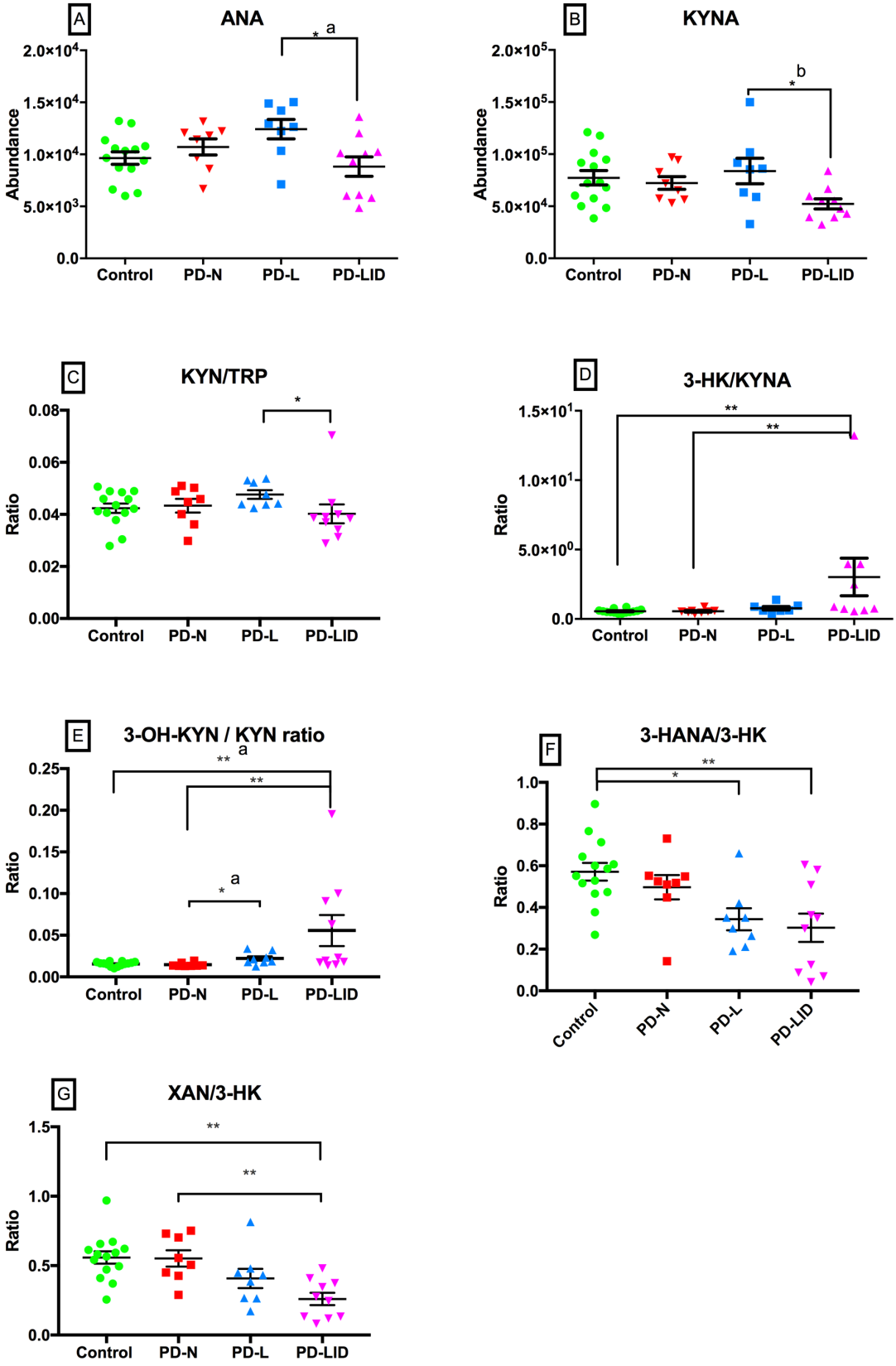

Figure 22: Group differences in plasma TRP metabolite levels and their ratios. Parametric data analysed with ANOVA and subsequent Bonferronis multiple comparisons, non-parametric data analysed with Dunn's test with

Bonferronis correction for multiple comparison. (A) ANOVA, p=0.0265 (B) ANOVA, p=0.0415 (C) Kruskal-Wallis, p=0.0479 (D) Kruskal-Wallis, p=0.0008 (E) Kruskal-Wallis, p=0.0038 (F) ANOVA, p=0.0024 (G) ANOVA, p=0.0005.

a: Not significant when excluding DLB and AD patients.

b: Only significant when excluding DLB and AD patients.

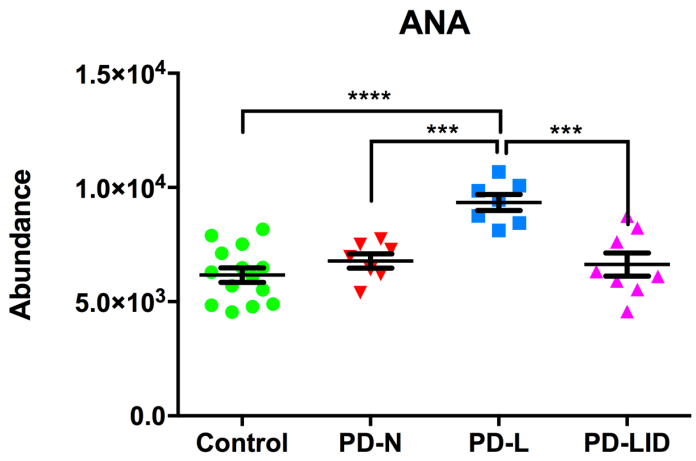

Figure 23: CSF levels of ANA in groups. ANOVA, p=0.000. Bonferronis multiple comparison.

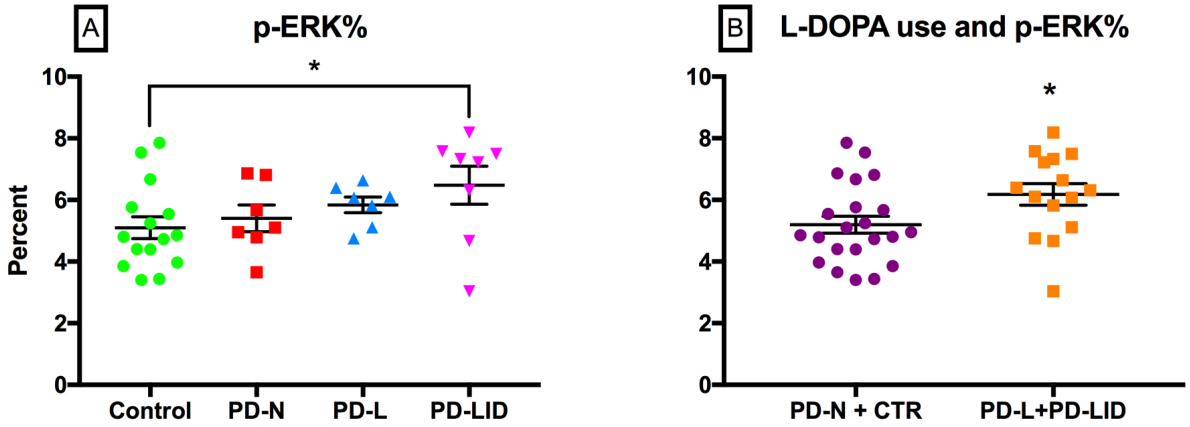

Figure 24: Percentage of phosphorylated ERK 1/2 compared to total ERK1/2 levels in CSF and the effect of L-DOPA on p-ERK%. (A) ANOVA, p=0.5846. Multiple comparison with unprotected Fischer's least significant difference (LSD) (B) Student's t-test, p=0.0318

CHAPTER 8: DISCUSSION

8.1 Diagnostic biomarker candidates

8.1.1 Alpha-synuclein:

In this project we have used both western blot and ELISA analyses for alpha-synuclein. Results are not significant when using ELISA analysis for t-α-syn in CSF, in line with other ELISA studies(Trupp *et al.* 2014, Aerts *et al.* 2012, Foulds *et al.* 2012, Tokuda *et al.* 2010). One study(Nielsen *et al.* 2014) was wrongly cited in our review(Andersen et al. 2016) as showing no difference between PD and controls. On the contrary, their results, published in another paper(Wennstrom *et al.* 2013), together with other ELISA studies(Fernandez et al. 2013, Mondello *et al.* 2014, Parnetti *et al.* 2014) have found significantly lower total α-syn levels in PD CSF. Also studies using time-resolved Förster's resonance energy transfer assay (TR-FRET)(van Dijk *et al.* 2014) and the more sensitive luminex assays (Hall *et al.* 2012, Hong et al. 2010, Shi *et al.* 2014) found significantly lower levels in PD CSF. These discrepancies, it could be argued, might be the result of several methodological differences regarding sampling procedures, sample processing prior to storage, and the use of different capturing antibodies.
A study has shown that erythrocytes contain the majority of α-syn in blood, and a mean erythrocyte α-syn concentration of 30 µg/ml in our study is in line with their results(Barbour *et al.* 2008). The results also reveal the markedly higher concentration of α-syn in blood compared to CSF, with a factor of approximately 10^6. Focusing on o-α-syn in the periphery instead of total α-syn seems to be a better approach with one study finding a significantly increased o-α-syn compared to total blood protein in PD compared to controls (Wang *et al.* 2015). In conclusion it seems debatable whether measuring total α-syn by itself is an effective diagnostic tool or not.

Focusing on the western blot α-syn analyses, it is interesting that PD patients have lower CSF levels of monomeric α-syn phosphorylated at the serine 87 residue (p-ser87-α-syn). One would expect PD patients to have higher levels of modified α-syn species, as was the case in one study that found increased levels (albeit with overlap) of α-syn phosphorylated at the serine 129 residue (p-ser129-α-syn) in PD CSF (Majbour *et al.* 2016). On the other hand it may be that aggregation of alpha-synuclein, especially phosphorylated α-syn, is more

pronounced in PD CSF. Initially our western blot analyses do not show an increase in oligomeric α-syn (dimeric, tetrametic), but since there may be other factors than disease status affecting levels of t-α-syn (and therefore also levels of modified and aggregated α-syn), comparing oligomeric α-syn to the amount of monomeric α-syn may take that variation into account. This seems to be supported by our findings. Patients have a significantly higher ratio between tetrametic and monomeric p-s87-α-syn as well as tetrameric α-syn and monomeric α-syn visualized with the 5-G4 ab. Other studies also find that the ratio between t-α-syn and modified or aggregated α-syn increases the discrepancy between PD and controls, such as an increased o-α-syn/t-α-syn-ratio (Tokuda et al. 2010, Majbour et al. 2016, Parnetti et al. 2014) or an increased p-s129-α-syn/t-α-syn ratio(Majbour et al. 2016). But in contrast to the findings of the two aforementioned studies and another ELISA study(Park *et al.* 2011), we did not find significantly increased CSF levels of oligomeric α-syn (such as dimeric and tetrameric conformations). It may be due to the low number of observations and the fact that the other studies used ELISA instead of western blot analyses.

8.1.2. Oligomeric protein analysis:

In our L-Tryptophanol manuscript (Appendix III), the findings of the Trol assay are discussed in detail.

It has to be noted that the Trol assay seems to bind to prefibrillary versions of Aβ and other amyloids, but not α-syn (Reinke et al. 2010). So it seems that the secondary, tertiary and quaternary structure rather than the primary structure of the protein is more important for the affinity of the indole marker used in the Trol assay.

CSF Trol:

I argue that lower CSF concentrations of oligomeric proteins might be due to the aggregation of oligomeric proteins in CNS neurons of PD patients. The argument here is the same as with a decrease of total α-syn due to aggregation. Potentially, a decreased bulk flow through the brain parenchyma would decrease the clearance of prefibrillary proteins to the CSF. I also argue that L-DOPA treatment itself might induce aggregation of oligomeric proteins, since it is known that the toxic L-DOPA metabolite DOPAL increases aggregation of α-syn into oligomeric shapes(Burke *et al.* 2008). But, there is no significant difference between PD-N and

L-DOPA-treated patients in terms of CSF Trol, and the L-DOPA daily dose does not correlate with CSF Trol.
When excluding the DLB patient, CSF Trol negatively correlated with disease duration, but not the use of L-DOPA. A gradual decrease of CSF Trol could then represent the successive increase in protein aggregation with further deterioration, and it could be speculated whether CSF Trol scores could be used as a rate marker.

Plasma Trol:
The significantly higher plasma Trol scores may be due to peripherally affected nerve cells releasing oligomeric proteins, since they are in direct contact with the blood stream. Alternatively a breakdown of the BBB may release these proteins into the bloodstream from the CSF. It may be, that other amyloids follow the same dynamic as α-syn: We know that CNS derived exosomes containing α-syn can be measured in plasma of PD patients(Shi et al. 2014), and that o-α-syn can be measured in plasma(Pchelina *et al.* 2017). This notion seems unlikely though, since a breakdown of the BBB would also be expected to increase the influx of oligomeric proteins to the CSF, hypothetically increasing CSF Trol scores in PD.
Few studies on plasma oligomeric α-syn have been performed. One ELISA study found increased levels of o-α-syn in PD patients, the test having a specificity of 0.852, sensitivity of 0.529 and PPV of 0.818(El-Agnaf *et al.* 2006). Conversely, another ELISA study on drug naïve patients did not find this increase, arguing that anti-parkinsonian treatment itself might affect the conformation of α-syn(Park et al. 2011). Perhaps the same could be true for other amyoid species. One major confounder may lie in the selection of PD patients. GBA mutations, as written earlier, are the most common genetic cause for PD development. In one study, directly separating sporadic PD patients from PD patients with GBA mutations, a significant increase of p-o-α-syn (and decrease of plasma GCase activity) compared to controls was only found in patients with known GBA mutations, and not in sporadic PD(Pchelina et al. 2017). Maybe the peripherally decreased GCase activity in these patients has more profound systemic effect, explaining the increased peripheral production of o-α-syn. If the selection of PD patients with random GBA mutations is not uncommon, this could lead to a decreased p-o-α-syn in a selected PD population, but would not be a shared feature of all patients. Perhaps affected GCase activity not only affects α-syn conformation, but also the conformation of other amyloids.

Decreased CSF Trol/plasma Trol ratio in PD:

It could possibly be argued that the combination of central aggregation of oligomeric proteins in PD, combined with the peripheral production of oligomeric proteins in the PNS could decrease the CSF Trol/plasma Trol ratio significantly. Alternatively it might reflect a larger escape of oligomeric proteins through a leaky BBB, although it could be expected that proteins might as well travel the opposite direction.

Decreased CSF Trol/total CSF protein ratio:

The BBB is affected in advanced PD with a possible influx of plasma proteins(Pisani *et al.* 2012). Assuming that the decrease of CSF Trol in PD is due to the aggregation of oligomeric proteins intraneuronally, accordingly, the combination of an increased influx of plasma proteins and intraneuronal oligomeric protein aggregation could explain the significantly decreased CSF Trol/ total CSF protein ratio.

8.1.3 Glucocerebrosidase activity:

In our study we found significantly decreased GCase activity in PD CSF. It has been argued that to minimize the potential effect on GCase activity due to a potentially defective BBB, this should be corrected for by dividing the GCase activity with the total CSF protein (van Dijk et al. 2013). When doing so, our GCase assay performs very well in separating PD from controls, also when excluding the DLB and AD patients. I identified three studies using the same substrate for the fluorescence assay, with one study finding no difference between patients and controls (van Dijk et al. 2013), and two studies also finding a decrease in GCase activity(Balducci *et al.* 2007, Parnetti et al. 2014). I am not sure as to why there seems to be a discrepancy. One noticeable difference is the sample processing. Whereas Balducci et al collected the CSF in iced tubes and we kept the collected CSF in ice water until centrifuging, both also processing the CSF and freezing it within 30 minutes and Parnetti et al immediately freezing the samples, Van Dijk did not keep the CSF cool until processing and had a 2 hour limit. It has been shown that GCase activity significantly declines within a couple of hours from LP if the sample is kept at RT, whereas storage at 4°C prior to freezing allows for a broader time span prior to freezing without affecting GCase activity significantly (Persichetti *et al.* 2014).

Nevertheless, our results do point to a significantly decreased GCase activity in a group of PD patients, potentially indicating that affection of GCase activity in CSF is a common feature among patients. Decreased GCase activity has been shown in post-mortem obtained SN and cerebellum of both PD patients and PD patients with GBA mutations(Gegg et al. 2012). Studies of blood plasma from PD patients with GBA mutations has been linked to significantly lower plasma GCase activity compared to controls, whereas PD patients with no known GBA mutation did not have decreased GCase activity(Pchelina et al. 2017, Ortega *et al.* 2016). A larger study did find a decrease in GCase activity in blood from PD patients compared to controls, even when excluding patients with known GBA mutations and a clinical history indicating other mutations linked to PD(Alcalay *et al.* 2015). This could point to decreased GCase activity being a part of the pathophysiology in idiopathic PD. In a study that found decreased CSF GCase activity in PD they did not find a significant difference in PD patients with and without GBA1 mutations (Parnetti et al. 2014). Comparing it to the studies finding significantly decreased plasma GCase activity only in PD with GBA1 mutations, this could point to a difference in the way the pathophysiological processes involved in PD affect CNS and plasma GCase activity.

It has to be noted that even though patients with Gaucher's disease type 1 have a significantly higher risk of developing PD compared to the background population, only a smaller percentage will develop PD(Rosenbloom *et al.* 2011). If decreased GCase activity by itself is not enough for the development of PD, other pathophysiological pathways involved in PD may simultaneously affect GCase activity. Even though we do not have a genetic analysis on our patients, with a decrease in GCase activity among the entire group, it seems unlikely that GBA mutations account for all the patients with decreased GCase activity. I previously mentioned a study showing that o-α-syn may negatively affect GCase activity(Mazzulli et al. 2011), and this could be a possible link between idiopathic PD and decreased GCase activity. Supporting the notion, one study also found patients with DLB to have lower CSF GCase activity than controls and patients with AD and frontotemporal dementia(Parnetti *et al.* 2009). This could indicate that GCase dysfunction is a common feature among synucleinopathies. It is worth mentioning that the DLB patient in this project had the lowest GCase activity of all. GBA1 mutations are even more clearly linked to DLB development than PD(Nalls et al. 2013), and even though it is speculative, one might suspect that this patient could be the carrier of a GBA1 mutation.

There are methodological differences in our analysis that require mentioning and that may have affected our results. Due to a sudden breakdown of the previously used fluorescent plate reader, some of our samples were analysed using a plate reader from a different brand. For this reason, to compare results, all samples were normalized by dividing them with the control mean of the different assays.

8.1.4. Tryptophane metabolites:

In our LC-MS based metabolomics study we have not yet performed detailed analysis of all detected metabolites. So far our only results are the ones related to TRP metabolism (see fig.24). Several studies have assessed a wider spectrum of plasma, serum, and CSF metabolites (Roede *et al.* 2013, Wuolikainen *et al.* 2016, Bogdanov *et al.* 2008, Johansen *et al.* 2009, Lewitt *et al.* 2013, LeWitt *et al.* 2017, Hatano *et al.* 2016, Trupp et al. 2014, Ascherio *et al.* 2009), identifying changes in the purin, TRP, and tyrosine metabolism (Ascherio et al. 2009, Trupp et al. 2014, Lewitt et al. 2013, Johansen et al. 2009, Hatano et al. 2016), as well as identifying metabolites with a prognostic value with regards to rate of disease development (LeWitt et al. 2017, Roede et al. 2013). Contrary to studies finding decreased TRP in serum (Hatano et al. 2016) and CSF (Trupp et al. 2014), we did not find decreased CSF or plasma TRP. Differing from our study, Trupp et al only included newly diagnosed untreated PD patients, and Hatano et al used serum samples. It is known that L-DOPA can affect tyrosine metabolism(De Deurwaerdere *et al.* 2016), but L-DOPA can also affect TRP metabolism. L-DOPA has been shown to compete with TRP for uptake by an amino transporter in serotonergic neurons. L-DOPA also competes with TRP for the amino acid decarboxylase (AADC) enzyme. The effect is a decreased serotonin production due to decreased TRP uptake and metabolism (Borah & Mohanakumar 2007). Serotonin production may also be affected be the concurrent use of AADC-inhibitors with L-DOPA treatment (such as Sinemet®).

As described in our article (Havelund et al. 2017)(Appendix III) the other degradational pathway for TRP is the KYN pathway in which TRP is converted into KYN by the enzymatic activity of indol 2,3-dioxygenase (IDO) and kynurenine formidase (fig.25). KYN may be transformed into ANA (due to kynureninase activity), KYNA (due to kynurenine transaminase activity (KAT)) or 3-HK (due to kynurenine 3-monoxygenase activity (KMO)). The metabolites in this pathway are neuroactive and are related to psychiatric disorders such as schizophrenia

(Oxenkrug *et al.* 2016, Fazio *et al.* 2015, Kegel *et al.* 2014, Chiappelli *et al.* 2014) and bipolar disorder (BD)(Lavebratt *et al.* 2014, Olsson *et al.* 2012, Sellgren *et al.* 2016, Savitz *et al.* 2015, Birner *et al.* 2017). In general terms the pathway leading to KYNA production is called the neuroprotective branch, whereas the pathway leading to 3-HK and QUIN production is called the neurotoxic branch(Campbell *et al.* 2014). A correlation between MOCA scores and plasma KYN could indicate an expected correlation between the KYN pathway and cognitive performance. One reason for the direct correlation between plasma KYN and MOCA scores may be a decreased availability of TRP for KYN production. If L-DOPA treatment decreases the availability of TRP (patients with the highest LED have the lowest MOCA scores), it may also decrease serotonin production, which could affect cognitive performance.

We identified significantly increased CSF ANA in PD patients with the same pattern in plasma, albeit not significant. ANA easily crosses the BBB by diffusion, whereas KYN and 3-HK are transported actively across the BBB by the large neutral amino acid carrier(Fukui *et al.* 1991). This implies that the blood pool of these substances, opposite of 3-HANA, KYNA and QUIN, contribute to the CSF pool. Whereas increased ANA levels have been measured in synovial fluid of patient with rheumatoid arthritis(Igari *et al.* 1987) and in plasma of patients with type 1 diabetes mellitus(Oxenkrug *et al.* 2015), no studies have previously found increased ANA in PD CSF. One potential source for this change may be related to differences in the intestinal microbiome. Some bacterial strains are able to produce ANA to be used as a precursor for TRP production (Rydon 1948), so changes in the composition of intestinal bacteria may affect ANA levels. The production of ANA is done by the enzyme kynureninase (KYNU). Since PD patients have increased CSF ANA this could indicate an increased KYNU activity. Our results show that plasma ANA levels and plasma 3-HANA/3-HK ratios are significantly correlated in PD, which supports the notion of increased KYNU activity. Recently, a study has shown significantly increased KYNU activity compared to IDO activity in keratinocytes from patients with the inflammatory skin disease psoriasis(Harden *et al.* 2016). This would drive the TRP metabolism towards the production of ANA and 3-HANA, and in the end QUIN. The same study found that exposing cells to a psoriatic milieu with interleukin 17, interferon ϒ, and tumor necrosis factor α, increased the activity of both IDO and KYNU. It is proposed that KYN may act as an immunosuppressant, but that increased KYNU activity drives the KYN metabolism in a proinflammatory direction; especially since 3-HANA was shown to increase the expression of proinflammatory genes in different human cell types(Harden et al. 2016).

Our findings could point to an interesting common pathogenic pathway between inflammatory diseases. The constant proinflammatory activity may have long-term deleterious effects on neurons due to ROS production and microglial activation. Furthermore, it seems that psoriasis patients actually do have a significantly increased risk of developing PD, with a meta analysis showing a 38% increased risk of PD in psoriasis patient compared to subjects without psoriasis(Ungprasert *et al.* 2016). In our study, PD patients have significantly lower 3-HANA/3-HK ratios than controls, indicating that they might have lower KYNU-activity. But, 3-HANA was the only variable of the TRP metabolites significantly increased in non-fasting controls compared to fasting controls. And since a larger subset of controls was fasting, this is a plausible explanation for the increased 3-HANA/3-HK ratios in controls. ANA was not significantly affected by fasting status in controls.

Our finding of an increased plasma 3-HK/KYNA ratio in PD patients also point to a KYN metabolism towards the neurotoxic branch, with a potentially increased KMO activity compared to KAT activity in PD versus controls. The same pattern was found in another CSF study (Lewitt et al. 2013). A post-mortem study also found increased levels of 3-HK in the putamen and SN of PD brains as well as a reduced KYN/3-HK ratio (Ogawa *et al.* 1992). Hypothetically, changing enzyme activity inducing a higher KAT/KMO activity ratio may provide neuroprotection. Using the KMO-inhibitor Ro 61-6048 it has been shown in animal studies that serum KYNA concentrations are raised acutely (Samadi *et al.* 2005, Gregoire et al. 2008). The same studies showed that the KMO-inhibition did not negatively affect the effect of L-DOPA treatment in animals. It has to be noted that the difference in 3-HK/KYNA is largely driven by an increase in PD-LID, and the role of KYNA and KMO-inhibition in LID will be further elaborated in the discussion section **7.2.1**.

The significantly lower CSF 5-HIAA levels in PD (Fig.20B) could be related to the L-DOPA treatment in PD-L and PD-LID, which potentially decreases uptake and metabolism of TRP in serotonergic neurons.

The negative correlation between CSF ANA levels and MOCA scores could indicate that KYN metabolism towards QUIN has a negative impact on cognition. This would be in line with findings linking an increased production of QUIN with neurocognitive deficits such as dementia in HIV patients (Kandanearatchi & Brew 2012).

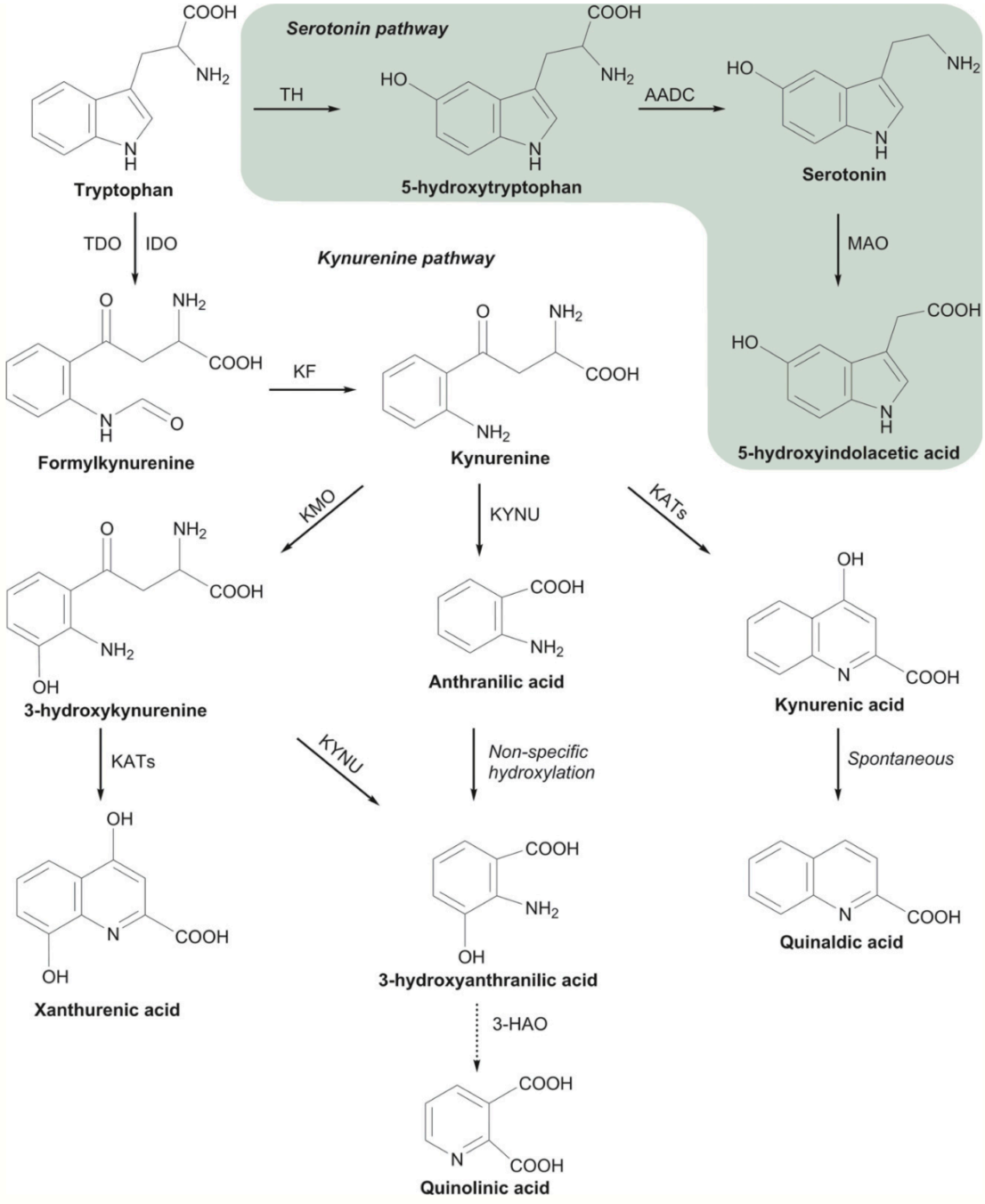

Figure 24: Metabolic pathways for tryptophane. TDO; tryptophan 2,3-dioxygenase, IDO; Indoleamine- 2,3-dioxygenase, KF; kynurenine formamidase, KMO; kynurenine 3-monooxygenase, KYNU; kynureninase, KATs; kynurenine aminotransferases, 3-HAO; 3-Hydroxyanthranilic acid dioxygenase, AMO; anthranilate 3-monooxygenase, TH; tryptophan hydroxylase, AADC; aromatic amino acid decarboxylase, MAO: monoamine oxidase.

8.1.5 Final thoughts on diagnostic biomarker candidates:

The marked decrease of the CSF Trol/ CSF total protein ratio across the three PD groups indicates that we have identified a potential diagnostic biomarker candidate, corroborated by the good performance of this ratio in the ROC. Of course these findings have to be repeated in much larger scale to take into account random variation. The strength of this ratio seems also to be indicated by the lack of confounding due to age, gender, or fasting status. Ultimately, we do not know which specific proteins are actually measured by the L-Tryptophanol fluorescence assay, meaning that we do not know whether it Aβ, or any other amyloid that can also conform into β-sheet-configurations, and it is only a hypothesis that intraneuronal aggregation is actually the reason for the decrease in CSF Trol. Pragmatically, if findings can be replicated, the proposed mechanism behind the measurable changes is irrelevant from a clinician's viewpoint, when focusing on diagnostics.

8.2 L-DOPA induced dyskinesia biomarker candidates

8.2.1 Tryptophane metabolism:

Focusing on TRP metabolites, we notice some specific changes in PD-LID compared to PD-L: Dyskinetic patients in our study have significantly lower CSF and plasma ANA than non-dyskinetic patients, contrary to the pattern seen in PD total compared to controls. At the same time, dyskinetic patients also have lower plasma KYN/TRP ratios, and when excluding the DLB patient, dyskinetic patients have significantly lower plasma KYNA than non-dyskinetic patients.

As discussed in the previous section, increased KYNU activity (responsible of producing ANA) may be related to a proinflammatory state. Following that line of reasoning, our findings might point to an increased proinflammatory state in non-dyskinetic patients. This seems to counter a major review indicating that neural inflammation conveyed by microglia and astrocytes is actually related to development of LID(Carta *et al.* 2017). It may of course be that other factors affect plasma ANA levels such as the availability of KYN, and potentially an increased activity of KAT or KMO compared to KYNU is responsible. As will be discussed now, our other findings may conversely corroborate the notion of inflammation being involved in LID development.

In our study, dyskinetic L-DOPA treated patients have significantly lower plasma KYNA levels than non-dyskinetic patients. This may indicate a decreased KAT activity or that KYN is simply more rapidly being converted into e.g. 3-HK. Four of the dyskinetic patients had a marked increase in plasma 3-HK driving the significant difference between PD-LID and controls as well as PD-N, in terms of an increased plasma 3-HK/KYNA ratio, increased plasma 3-HK/KYN ratio and decreased plasma XAN/3-HK ratio. Both KYNA and XAN are produced by KAT activity. Thus, the decreased KAT activity combined with an increased KMO activity would hypothetically explain the differences. The NMDA receptor subunit NR2B is abundant in the striatum and is among many different functions related to long term potentiation, learning and memory(Loftis & Janowsky 2003), and a study found an increase of NR2B-containing NMDA-receptors in dyskinetic MPTP-treated non-human primates(Ouattara *et al.* 2009). As mentioned previously, glutamatergic receptors play a great role in the development of LID, in some part linked to the TRP metabolism. The two TRP degradational pathways, the

neuroprotective branch (KYNA) or the neurotoxic branch (3-HK and finally QUIN), seem to have opposing effects on NMDA receptors. KYNA binds to the glycin binding site on the NR1 NMDA subunit(Parsons *et al.* 1997) and an *in vivo* study on cortical neurons showed that KYNA effectively diminishes the agonistic effect of QUIN on NMDA receptors(Perkins & Stone 1982). Further supporting the involvement of glutamatergic receptors in LID, studies have looked at the effect of the KMO-inhibitor Ro 61-6048, which raises KYNA levels acutely. Using Ro 61-6048 on a group of L-DOPA treated MPTP-treated primates showed a 20% decrease in LID (Samadi et al. 2005) as well as a delayed LID development (Gregoire et al. 2008), potentially related a prevented increase of preproenkephalin in the striatum (Tamim *et al.* 2010). Thus, the increased 3-HK/KYNA ratios in PD-LID plasma (with the same pattern being noticeable in CSF) could suggest increased glutamatergic transmission in the basal ganglia as a cause for LID. This provides an interesting treatment prospect for LID in PD. Would it be possible to treat dyskinetic patients with such a KMO-inhibitor, which seems to have marked anti-dyskinetic effects? Whether the antidyskinetic effect is actually due to NMDA-receptor inhibition is debated, since an *in vivo* animal study has shown that Ro 61-6048 induced increased levels of CSF KYNA is not enough to antagonize NMDA receptors(Obrenovitch & Urenjak 2000). KYNA also works as a slow onset voltage-independent noncompetitive antagonist of the α7 nicotinic acetylcholine receptor (α7-nAChR)(Hilmas *et al.* 2001). The α7-nAChR is highly involved in synaptic plasticity(Udakis *et al.* 2016), and changes in synaptic plasticity may explain the anti-dyskinetic effect of KYNA. Alternatively, it may simply be due to a decreased DA release in striatum: Striatal cholinergic interneurons can induce DA release in the striatum due to activation of nicotinergic receptors, regardless of action potentials in the dopaminergic neuron(Threlfell *et al.* 2012). The idea of an anti-dyskinetic effect of nAChR-inhibition seems to be contradictory to other findings: It has been argued that neuroinflammatory processes involving microglia and astrocytes may be related to LID development, and that the anti-inflammatory potential of ACh agonists in astrocytes is due to α7-nAChR stimulation(Carta et al. 2017). The α7-nAChR agonist 5-(6-((3R)-1-azabicyclo(2,2,2)oct-3-yloxy)pyridazin-3-yl)-1H-indole (ABT-107) has been shown to provide a long term LID depression in MTPT and L-DOPA treated non-human primates (Zhang *et al.* 2014). A partial α7-nAChR agonist (R)-3-(6-r-Tolyl-pyridin-3-yloxy)-1-aza-bicyclo(2.2.2)octane (AQW051) significantly reduced LID without decreasing the antiparkinsonian effect of L-DOPA and even prolonging the effect of the L-DOPA on MTPT

treated non-human primates(Di Paolo *et al.* 2014). It may be that the combined effect of KYNA on both glutamatergic and acetylcholinergic receptors is involved in the antidyskinetic effects due to other mechanisms than the antidyskinetic effect of direct α7-nAChR activation. Or maybe prolonged exposure to α7-nAChR agonists induces desensitization or downregulation of nAChR, the net effect being decrease nicotinergic stimulation.
As a final comment, there seems to be an interesting coupling between LID in PD and affective disorders in terms of changes in the TRP metabolism. The pattern of an increased 3-HK/KYNA ratio has also been shown in patients with bipolar disorder(Birner et al. 2017). It may be that changes in KYN metabolism are also involved in changes in dopaminergic transmission. Much evidence points to a hyperdopaminergic activity in bipolar disorders, especially in manic phases characterized by lack of impulse control(Ashok *et al.* 2017). Interestingly, it seems that impulse control disorders such as punding significantly correlates with LID in PD(Silveira-Moriyama *et al.* 2006). The risk of tardive dyskinesia due to neuroleptic treatment seems to be increased in bipolar disorders(Mukherjee *et al.* 1986), and it could be speculated that similar mechanisms are involved in the two types of dyskinesia.

8.2.2. Catecholamines:

As mentioned in the methods section, when writing the paper on CSF catecholamines(Andersen et al. 2017), we did not use Bonferroni adjusting for multiple comparisons. It could be argued that Bonferronis adjustment, especially in the light of few observations, may increase the risk of type II errors(Perneger 1998).
Previously in this thesis I wrote about the potential causes for LID, including a more rapid L-DOPA turnover as well as a non-dopaminergic L-DOPA metabolism. PD-LID has a higher mean CSF DA than PD-L and a more marked increase of DA compared to controls and PD-N. Combined with a significantly increased DA/L-DOPA ratio in PD-LID compared to PD-L, we argue that these changes may be due to the combination of an increased neuronal release of DA as well as an accelerated decarboxylase activity, converting L-DOPA to DA. This could indicate a less controlled non-dopaminergic L-DOPA metabolism and DA release, e.g. from 5-HT terminals lacking an autoregulatory feedback mechanism (Carta et al. 2007), which might

increase extracellular DA levels in striatum, affecting sensitized D1 receptors. It is also argued that a decrease in DAT on the remaining striatal terminals(Sossi *et al.* 2007, Sossi *et al.* 2009) would compromise the reuptake of released DA. PD-LID has significantly lower DOPAC/DA ratios than PD-L, further indicating non-dopaminergic L-DOPA uptake and release since DOPAC is made from DA in dopaminergic neurons by monoamine oxidase activity. The increased MHPG/NA ratio in PD-LID compared to PD-L could indicate a significantly higher NA turnover in dyskinetic patients. A study has found an increased firing rate in the locus coeruleus in dyskinetic 6-hydroxydopamine lesioned rats(Miguelez *et al.* 2011), and it could be speculated whether an increased NA turnover is a general feature of dyskinetic patients.

8.2.3: Phosphorylated extracellular signal-regulated kinase

The significantly increased percentage of p-ERK 1/2 in dyskinetic patients could support the hypothesis that an increased D1 receptor stimulation increases the cAMP-induced phosphorylation of ERK 1/2. That L-DOPA has a marked effect on the phosphorylation of ERK 1/2 is also supported by the fact that L-DOPA treated patients have significantly higher p-ERK% than PD-N and controls combined.

8.2.4: Final thoughts on dyskinesia biomarker candidates:

In this thesis I have discussed the potential correlation between changes in TRP and L-DOPA metabolism as well as p-ERK and LID. Future studies of course have to be made including a wider range of LID severities as well as larger patient groups to take into account random variance. Since LID is a very dynamic process, dynamic changes in levels of several LID biomarker candidates might be expected. With many variables accounting for the rate of intestinal uptake of L-DOPA, this does pose a challenge in terms of timing the lumbar puncture and blood sampling with L-DOPA intake. Ideally, patients should be monitored after L-DOPA intake, not performing the sampling procedure at a fixed time interval after L-DOPA intake, but at the proposed peak of the patient's dyskinesia. The logistics of this study sadly

did not make this possible. Including more severely dyskinetic patients would also pose another challenge. Severe truncal dyskinesia but also dyskinesia of the extremities may prevent the patient from lying still. This would potentially make it too dangerous to perform a lumbar puncture.

The prognostic value of these LID biomarker candidates could also be assessed in longitudinal studies, sampling CSF from L-DOPA naïve PD patient just prior to commencing L-DOPA treatment. This may reveal whether differences in TRP or L-DOPA metabolism is a general feature in LID patients or changes due to L-DOPA treatment itself.

Assessing changes in LID biomarkers candidates in PD patients treated with DBS might also further our understanding of the mechanisms behind the antidyskinetic effect of DBS. Also PD patients going from oral L-DOPA to intestinal continuous Duodopa-treatment could be assessed, observing the potential dynamic changes in metabolism.

8.3 General discussion

One of the great challenges in this project was the recruitment of patients. Initially, we sought to include only patients who were interested in undergoing a lumbar puncture. Later, we began including patients who only wanted to provide blood samples for analysis. Although we included three other recruitment places we were not able to recruit more than 26 patients due to the overall schedule. Also control recruitment became a challenge, with only 16 controls being included in the study. The lack of subjects made it impossible to select patients and controls according to age or gender. There are two major confounders that we identified accordingly across many of our results: Age and gender. It becomes hypothetical whether age or gender did actually confound the results or whether disease status in a population of older predominantly male subjects compared to the control population created this correlation. Only by doing further analyses between gender and age matched groups is it possible to make that distinction. The small sample sizes also make it impossible to take into account more than two variables apart from disease status in a multiple regression analysis.

Given the small sample size, it is also prudent to view the results from our group analyses with caution. With sample sizes in the groups ranging from 7 to 16, random variation might have a marked effect on the results.

Whereas the PD population was fasting prior to the lumbar puncture, only 5 out of 16 controls were fasting, due to the recruitment procedure. Especially when focusing on metabolomics, we took the fasting status into account in the control group; but in our small control group it does not seem as if fasting significantly altered anything but CSF DOPAC and plasma 3-OH-anthranilic acid levels.

Due to practical issues, the timing of the lumbar puncture was delayed for many of the controls compared to the patients. We cannot rule out that diurnal changes in levels of the biomarker candidates may have affected the results.

Some substances, such as HVA, may be affected by which gradient of CSF is being used(Dhondt 2004). Even though I did not choose the level of lumbar puncture according to the height of the patients, the tapping procedure was quite similar among patients and controls. Approximately the same amount of CSF was drawn for both PD and controls for routine analysis prior to the sampling of CSF for special analyses. The same amount of CSF was drawn and directly frozen for catecholamine analysis, and subsequently the same amount was drawn in a single tube, gently mixed prior to processing and aliquoting, for all other analyses. In this way, to a certain extent, we have taken into account any gradient difference.

As mentioned previously, L-DOPA intake (and perhaps in general antiparkinsonian medication) may have unknown effects on many of our measured variables. Another consequence of not pausing medication is the clinical appearance of the PD patient. Even though I can only assume that PD patients included in the study are sufficiently treated, and even though the clinical rating was done at a fixed interval after L-DOPA intake, it would be expected that patients react differently time wise to the L-DOPA dose prior to examination. The ON-stage motor performance of a single patient compared to the mean ON-stage motor performance of all L-DOPA treated patients might not reflect that patient's OFF-stage performance compared to the mean OFF-stage motor performance of all L-DOPA treated patients. This could potentially mask any correlation between motor performance and the assessed biomarker candidates. From an ethical perspective, I did not find it appropriate to ask PD patients not to take their medication for a shorter duration. Apart from a potential marked decrease in daily function until the day of participation in the study, it may have made it more difficult for the patients logistically to arrive at the study center.

Unfortunately, the MDS-UPDRS did not exist in a Danish version when the project was begun. As noted, we made our own Danish translation of part I, II and IV. Given the lack of

verification of our translation, the otherwise quantifiable data on non-motor symptoms etc., was not to be used. As a side note it can be mentioned that the unverified total UPDRS score did significantly correlate with disease duration, whereas UPDRS part III did not.

Diagnostic uncertainties have to be mentioned. Some patients had had symptoms of Parkinson's disease for less than 5 years, and although patients included fulfilled the UKBBC and were diagnosed by specialists in movement disorders, there is always a risk of patients actually suffering from atypical Parkinsonian disorders. Some studies perform post-mortem neuropathological verification of all patients, which of course is not within the scope of this project.

It is evident that DA metabolites are significantly affected by L-DOPA intake. It cannot be ruled out that in general, antiparkinsonian treatment may affect other metabolic pathways.

Therefore, all results from this study have to be interpreted with caution.

Chapter 9: Conclusion

In this chapter I will briefly summarize the significant findings in this study.

9.1: Diagnostic biomarker candidates

1. PD patients had significantly lower CSF levels of full-length monomeric α-syn and an increased ratio between full-length monomers and tetramers.
2. PD patients had significantly lower Trol scores in CSF and significantly increased Trol scores in plasma. The ratio between CSF and plasma Trol scores more significantly separated PD from controls. Using the ratio between CSF Trol scores and the total CSF protein concentration resulted in an even better distinction between PD and controls.
3. CSF normalized GCase activity was significantly lower in PD. Further normalizing GCase activity according to CSF protein concentration provided a highly significant separation of patients and controls.
4. PD patients not treated with L-DOPA had significantly lower levels of DOPAC, DA, L-DOPA and HVA.
5. CSF Trol scores correlated with disease duration. MOCA scores correlated with plasma KYN and correlated negatively with CSF ANA.

9.2: L-DOPA induced dyskinesia biomarker candidates

1. Dyskinetic patients had significantly increased DA/L-DOPA ratios, decreased DOPAC/DA ratios, increased HVA/DA ratios, and increased MHPG/NA ratios.
2. In plasma, dyskinetic patients had significantly lower levels of ANA, KYNA. They also had increased KYN/TRP-, 3-HK/KYN-, and 3-HK/KYNA ratios as well as decreased 3-HANA/3-HK-, and XAN/3-HK ratios.
3. CSF ANA levels were significantly decreased in dyskinetic patients.
4. CSF p-ERK% was significantly increased in dyskinetic patients.

Chapter 10: Concluding remarks

As is the case in many other studies on CSF biomarkers, the lack of subjects included in this project is marked. The lumbar puncture, compared to the blood sampling procedure, poses a potential risk of CNS infections, bleedings affecting spinal nerve roots and severe headaches induced by the vertical pressure difference due to the dural puncture. Many patients thus fear the procedure (oftentimes mistaking it for a bone marrow biopsy). But when performed by a skilled practician, the procedure seems benign for most participants.

To my knowledge no movement disorder clinic in Denmark routinely performs lumbar punctures as part of the diagnostic check up on patients with suspected PD. In a clinical setting performing such a procedure should only be done when it serves a clinically sound purpose, justifying the otherwise infrequent but severe risks from the procedure. There may be arguments for doing so already: Low CSF levels of Aβ1-42 seem to be related to poorer cognitive performance(Leverenz *et al.* 2011, Alves *et al.* 2010, Compta *et al.* 2012, Montine *et al.* 2010), but also serve as a prognostic marker for cognitive decline and dementia development(Siderowf *et al.* 2010, Compta *et al.* 2013, Alves *et al.* 2014, Schrag *et al.* 2017). This could provide clinicians with a prognostic tool regarding the expected severity of the almost inevitable cognitive decline related to PD.

Performed by many researchers, longitudinal studies are of great use when focusing on PD. Apart from the increased diagnostic certainty through follow up, successive clinical ratings of both cognitive and motor performances may reveal a prognostic value of a biomarker candidate (as just mentioned regarding Aβ1-42). Including medically un-treated newly diagnosed PD patients for sample analyses would also remove the possible confounding effect of antiparkinsonian treatment, and later follow up could verify the PD diagnosis.

In the future, the inclusion of patients with APD would be of great value, since one of the major aspects regarding diagnostic difficulties is related to the similarity between PD and APD in the early stages. Since we have not included any patients with MSA, PSP or CBD we cannot assume that the changes measured in our patient group are specific for PD or even for synucleinopathies.

In the end, the essential question when focusing on diagnostics seems to be: why use CSF biomarkers? Much evidence, as described earlier, points to a prior involvement of more peripheral nerves, indicating that CNS involvement (and thus marked changes in CSF

biomarkers) occurs at a later stage. Would it be better to focus on biomarker candidates in the periphery, such as skin biopsies, urin analyses, imaging of the autonomic peripheral nervous system (such as cardiac MIBG scintigraphy)? It could be argued that the clinical picture of premotor PD might be even more diverse than the already heterogenous clinical appearance of motor PD, making it very difficult to identify these patients. Especially since they might not even contact a physician due to such diffuse symptoms. Therefore I do think that CSF biomarkers will continue to play a potential role in the diagnosis of PD, as well as advancing our knowledge of the CNS disease processes involved in PD.

Chapter 11: Acknowledgements

First and foremost, I would like to thank all the participating patients and controls. Without their help there would be no project. The participation of patients in research projects is pivotal to the further understanding of this disease, and I am grateful that they chose to endure a long day of spinal taps and clinical ratings!

Egon Stenager; as my main supervisor you have been a tremendous support during rough times. You were the one who introduced me to the possibility of doing a research project, helping me prepare all the practical issues. It continues to amaze me how quickly you are able to answer my many e-mails, and I hope that it has not been too strenuous! You have given me free reigns during the entire PhD project, letting me learn from all the inevitable mistakes that I made during three years of research. But I have always been confident that should any problem arise that needed your assistance; I could always count on you. Thank you for many interesting scientific discussions and for broadening my perspectives on life as a clinician and researcher.

Jan Bert Gramsbergen; thank you for being my co-supervisor. You let me have an office space at the Institute of Molecular Medicine, for which I am very grateful. Over the years we have had a great deal of engaging conversations on science and politics and shared many frustrations regarding temporarily dysfunctional lab equipment. You have always been good at thinking outside the box, constantly asking new questions leading to potentially new research projects and ideas. Your thoroughness and ability to notice the small, but important, details in our manuscripts has been a great help!

Michael Binzer, thank you for your assistance as my co-supervisor! When I needed to find more patients to be a part of my study, you found a great deal of patients willing to participate. Thank you for that! Also, thank you for a lot of good discussions regarding the project and help in finishing the manuscripts as well as training me in performing clinical ratings.

Morten Blaabjerg, thank you for also being my co-supervisor, helping me find many of the patients and controls for the project, as well as providing really good feedback on manuscripts and the study itself!

Helle Østerballe, thank you for putting such an effort into finding patients in Sønderborg willing to participate in my study!

Harald Floer, thank you for helping me getting into place in the dept. of Neurology in Sønderborg, assisting and providing good feedback during the study!

Matthias Bode, thank you for your valuable input and assistance regarding the study.

Troels W. Kjær, thank you for your great support introducing me to the Dept. of Neurology in Roskilde, pulling a lot of strings for me to be able to find patients for the project, as well as giving good feedback on the study!

Akram Kamal and **Helle Thagesen,** thank you for your assistance in finding patients willing to participate in the study!

Susanne Aabling, Rikke Rosengren, Lene Kromann, Yrsa Clausen and **Dea Meyer,** thank you for your help assisting with the lumbar punctures and sorting all the practical issues!

Tobias Sejbæk, thank you for lending me your centrifuge!

The Dept. of Clinical Biochemistry in Sønderborg, Esbjerg, Odense & Roskilde, thank you for your assistance with CSF analyses and centrifuges!

Nive Ilangkovan and **Donna Lykke Wolff,** thank you for some really good times. I enjoyed starting the PhD club in Sygehus Sønderjylland together with you! Also thank you to everyone at the research facility for many good discussions and scientific input!

Jesper Havelund, thank you for the cooperation making the Kynurenine-article!

Glynn Jones, thank you for a great time in Aberdeen, inspiring talks, and for cooperation with the Trol assay!

Nils J. Færgeman, thank you for your assistance during the project

Niels H.H.Heegaard (1959-2017), in memory of Niels, helping me with analyses and article writing, always with a constant positive attitude. R.I.P.

Ulla Damgaard Munk, Dorte Lyholmer and **Signe Andersen,** thank you very much for your assistance in the lab!

Institute of Molecular Medicine, Neurobiology research, a big thank you to everyone at the institute! I have enjoyed our many scientific and non-scientific talks, drinking Friday beers with you all, eating copious amounts of cake and enjoying delicious food in AMNESIA!

Elsebeth Stenager, thank you for letting me be part of planning the summerschool in neuroscience! It has been a really good experience!

I am affiliated with **Odense Patient data Exploratory Network (OPEN), OUH**, who provided assistance for building my REDcap database used as a safe sharepoint for patient data. They also provided great assistance regarding sampling procedures, thank you!

I want to thank **Udo Kiscka** and **Helen Kennerley** for inspiring me to dive into the field of neurology and psychology. Thank you for good times in Oxford together with Eva and Noah!

To my dear parents, thank you so much for your constant love and support; your work as physicians and your dedication to your patients has always served as an inspiration to me.

This project has been supported by:

Region Syddanmark

Sygehus Sønderjylland

Syddansk Universitet

Knud og Edith Eriksens Mindefond

Parkinsonforeningen

A.P. Møller og Hustru Chastine Mc-Kinney Møllers Fond til almene Formaal, Fonden Til Lægevidenskabens Fremme,

Danske Banks Fond

Michael J. Fox Foundation

Overlægerådets Fællsfond, Læge Else Poulsens Mindelegat

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APPENDIX I

Paper 1

Acta Neurologica Scandinavica

Acta Neurol Scand DOI: 10.1111/ane.12590

ACTA NEUROLOGICA
SCANDINAVICA

Review Article

Cerebrospinal fluid biomarkers for Parkinson's disease – a systematic review

Andersen AD, Binzer M, Stenager E, Gramsbergen JB. Cerebrospinal fluid biomarkers for Parkinson's disease – a systematic review. Acta Neurol Scand: DOI: 10.1111/ane.12590.

Diagnosis of Parkinson's disease (PD) relies on clinical history and physical examination, but misdiagnosis is common in early stages. Identification of biomarkers for PD may allow early and more precise diagnosis and monitoring of dopamine replacement strategies and disease modifying treatments. Developments in analytical chemistry allow the detection of large numbers of molecules in plasma or cerebrospinal fluid, associated with the pathophysiology or pathogenesis of PD. This systematic review includes cerebrospinal fluid biomarker studies focusing on different disease pathways: oxidative stress, neuroinflammation, lysosomal dysfunction and proteins involved in PD and other neurodegenerative disorders, focusing on four clinical domains: their ability to (1) distinguish PD from healthy subjects and other neurodegenerative disorders as well as their relation to (2) disease duration after initial diagnosis, (3) severity of disease (motor symptoms) and (4) cognitive dysfunction. Oligomeric alpha-synuclein might be helpful in the separation of PD from controls. Through metabolomics, changes in purine and tryptophan metabolism have been discovered in patients with PD. Neurofilament light chain (NfL) has a significant role in distinguishing PD from other neurodegenerative diseases. Several oxidative stress markers are related to disease severity, with the antioxidant urate also having a prognostic value in terms of disease severity. Increased levels of amyloid and tau-proteins correlate with cognitive decline and may have prognostic value for cognitive deficits in PD. In the future, larger longitudinal studies, corroborating previous research on viable biomarker candidates or using metabolomics identifying a vast amount of potential biomarkers, could be a good approach.

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Key words: biomarker; cerebrospinal fluid; Parkinson's disease; Alpha-synuclein; metabolomics; Amyloid-beta; Tau-protein; neurofilament light chain

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Accepted for publication March 1, 2016

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, with a prevalence of 1% in the population older than 60 years (1). When the degeneration of dopaminergic neurons in the substantia nigra reaches 50–60%, the lack of dopaminergic input to the striatum causes the characteristic motor symptoms including hypokinesia, postural instability, rigidity and resting tremor. The diagnosis is overwhelmingly based on these clinical symptoms (2).

There is no established disease modifying treatment and the continuous progression of the disease results in increasing disability. After 2–5 years of treatment with monoamine oxidase inhibitors (MAO-B), dopamine agonists or dopamine substituting treatment with L-DOPA, the treatment gradually loses its efficiency. Patients will invariably develop significant daily fluctuations of symptom severity, and especially the treatment with L-DOPA increases the risk of developing potentially disabling involuntary movements, L-DOPA-induced dyskinesia (LID) (3).

It can be very difficult to diagnose PD and is almost impossible before the development of motor symptoms, because premotor symptoms, including olfactory deficiency, obstipation, sleep

disorders and depression, are very unspecific (4, 5). In earlier disease stages, other neurodegenerative diseases may mimic idiopathic PD. These atypical parkinsonian disorders (APD) include the synucleinopathies multiple system atrophy (MSA), Lewy body dementia (DLB) and the tauopathies progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) and have different prognoses and treatment responses. In a longitudinal study using neuropathologically confirmed PD as gold standard, Adler et al. (6) showed an 85% positive predictive value of the clinical diagnosis for patients with PD with >5-year disease duration. But the positive predictive value in patients responsive to drug treatment diagnosed within 5 years was only 53%, necessitating follow-up when including newly diagnosed PD patients in biomarker studies.

In summary, there is an urgent need for earlier diagnostics, differentiating between PD and other parkinsonian syndromes, a better understanding of PD pathogenesis and assessment of disease progression to evaluate the use of potential disease modifying drugs. There has been extensive reviews of the research in biomarkers, for example by Jimenez-Jimenez et al. (7). This review divides the biomarker candidates into six categories: A: neurotransmitters and neuromodulators, B: oxidative stress markers, C: inflammatory and immunological markers, D: growth factors, E: proteins involved in PD pathology and F: others. Under each category, we focus on their usability in these four clinical domains:

1. Distinguishing PD from controls
2. Distinguishing PD from other neurodegenerative diseases
3. Representing disease severity or cognitive abilities and
4. Being of prognostic value regarding disease severity and cognitive abilities.

Not all categories have biomarkers within these four domains. Only cerebrospinal fluid biomarker candidates are included.

Method

This study is based on a systematic literature search in the database PubMed performed 21 January 2015 using the search criteria: (cerebrospinal fluid biomarker) AND Parkinson's disease. Inclusion criteria were original articles on clinical studies on cerebrospinal fluid biomarkers including at least 20 patients with PD. Articles were excluded if only abstracts were available or if language of full article was not in English, and if the article was published before year 2000. Articles focusing on the development of new analysis methods were also excluded.

Of 295 articles identified in the database, 58 were included in this study (See Fig. 1 for details).

Results

Neurotransmitters and neuromodulators

In 2003, Braak et al. (8) showed that disease severity in PD can be neuropathologically staged, from premotor stages of the disease with Lewy body (LB) pathology starting in the olfactory bulb and dorsal motor nucleus of the vagus nerve in the medulla, gradually ascending to the pons, including the serotonergic neurons of the raphe nucleus and the noradrenergic neurons of the locus coeruleus, subsequently in stage 3 affecting the dopaminergic neurons of the substantia nigra in the ventral mesencephalon and in later stages areas of the neocortex.

PD vs controls – In line with the degeneration of catecholaminergic neurons, decreased concentrations of the dopamine metabolites dihydroxyphenylacetate (DOPAC) and homovanillic acid (HVA) as well as the noradrenergic metabolites dihydroxyphenylglycol (DHPG) and 3-methoxy-4-hydroxyphenylglycol (MHPG) have been observed in the cerebrospinal fluid (CSF) of patients with PD as compared to controls (see Table 1). Treatment with dopaminergic drugs, including levodopa, alters CSF-catecholamines and its metabolites (9). Therefore, catecholamines and its metabolites are best studied in drug-naïve patients or following L-DOPA washout. Accordingly, Goldstein et al. (9, 10) showed significant decreases in DOPAC, DOPA, noradrenaline (NA) and DHPG in patients with PD, including newly diagnosed PD patients. LeWitt et al. (11) found that CSF levels of HVA are not a reliable biomarker of PD, but the ratio of the purine metabolite xanthine over HVA seems a possible biomarker for patients with PD as compared to controls.

In a more recent study, LeWitt et al. (12) employed targeted metabolomics, using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS) and gas chromatography–mass spectrometry (GC/MS) on post-mortem obtained CSF from patients with PD and controls. In this study, 19 compounds were identified differing PD from controls including an increased ratio of the tryptophan/kynurenine metabolites 3-hydroxykynurenine (3-HK) vs kynurenic acid (KYNA). A change in the ratio

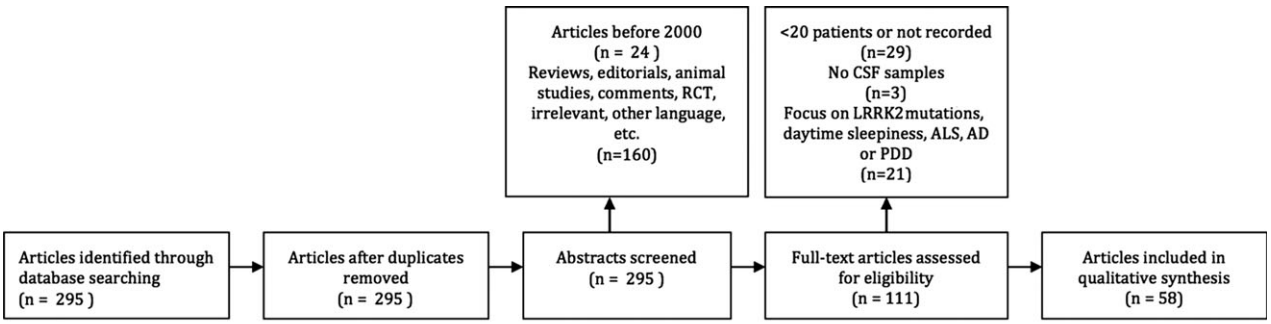

Figure 1. Flowchart of article selection process.

of these kynurenine metabolites is particularly interesting, as 3-HK may cause oxidative damage by general hydroxyl radicals and it is also a precursor of the excitotoxin quinolinic acid. KYNA is a broad-spectrum antagonist of glutamate receptors and has neuroprotective potential. Thus, an increased ratio of 3-HK/KYNA in the brain may promote neurodegeneration.

PD vs other neurodegenerative diseases – Contrary to Goldstein et al. (10) who found the catecholamine and metabolite concentrations to be similar in PD and MSA, Abdo et al. (13) found lower concentrations of MHPG, HVA and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in MSA patients with predominant parkinsonism (MSA-P), the latter explained with a more severe degeneration of neurons in the locus coeruleus in patients with MSA.

Disease severity and cognitive abilities – The xanthine/HVA ratio measured by LeWitt et al. (11) is significantly negatively correlated with clinical scores using the Unified Parkinson's Disease Rating Scale (UPDRS) part III (motor score).

Oxidative stress markers

Several PD disease mechanisms seem to induce oxidative damage in neurons. Oxidative damage through free radicals is linked to loss of antioxidative defensive capabilities, mitochondrial dysfunction, proteasomal dysfunction and microgliosis and definitely plays a role in PD, although it might not be the primary disease mechanism (14).

PD vs controls – A lack of antioxidative capabilities might make cells more susceptible for damage from free radicals. DJ-1 is a ubiquitous protein that through oxidation is thought to regulate transcription factors promoting an antioxidative defence (15). Mutations in the DJ-1 gene PARK7 are associated with hereditary recessive early-onset PD (16). Hong et al. (17) found a significant decrease in DJ-1 in patients with PD, but also found a positive correlation to α-synuclein (α-syn), age and the amount of blood contamination in the CSF. Waragai et al. (18) found an increase in CSF-DJ-1 in patients with PD (especially early PD), a finding which according to Hong et al. might be related to a different method of analysis (Western blot compared with the more sensitive Luminex assay) and not taking in to account blood contamination. Using an ELISA method, Herbert et al. (19) also found an increase in CSF-DJ-1, even when excluding samples with blood contamination. Hong et al. (17) argues that a decrease might be seen as a lack of adaptive response to the ageing process or a sequestration of DJ-1 with α-syn, whereas an increase is seen as a compensatory neuroprotective response (19). As the mean disease duration of the patients investigated by Hong et al. is higher than the mean disease duration of patients investigated by Herbert et al., opposing results might reflect a change in the amount of oxidative stress or response to oxidative stress in different disease stages, which seems to be the case for DJ-1 (see Table 3).

Glutathione is in its reduced form (GSH) one of the major antioxidative peptides, being converted to oxidized glutathione (GSSG). LeWitt et al. (12) found a decrease in GSSG in patients with PD, being interpreted as a decrease in antioxidative defence. Maarouf et al. (20) found increased glutathione S-transferase Pi activity in CSF of patients with PD, which might be a response to increased oxidative stress.

Silicon (Si) has the ability to bind aluminium (Al), which might be damaging to the central

Table 1 An overview of cerebrospinal fluid biomarker candidates for distinguishing between Parkinson's disease and controls

Biomarker candidate	Author (year) (reference number)	Cases/controls	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
A – Neurotransmitters and neuromodulators					
Anandamide	Pisani et al. (2010) (83)	56 (*de novo* 38, washout 8, L-DOPA-treated 10)/37	*De novo* 2.26, washout 4.39, L-DOPA-treated 2.76	↑ (*de novo* PD) ($P = 0.026$)	(UKBBC) (99)
DA, DHPG, DOPA, DOPAC, HVA, MHPG, 5-HIAA and NA	Goldstein et al. (2012) (9)	34/38	6.0 (1.0)	↓DOPAC ($P < 0.0001$) (early PD vs control): sens 100% spec 89% (PD vs control): sens 89% spec 80% ↓DHPG ($P < 0.0001$), ↓ L-DOPA ($P < 0.0001$), ↓NA ($P = 0.02$)	DOPA washout on all patients with PD to avoid effect of L-DOPA treatment on measurements (Diagnostic criteria not specified)
	Goldstein et al. (2008) (10)	77/87	9.0 (1)	↓ DOPAC and ↓DOPA ($P < 0.0001$ each)	Follow-up 6-[18F]-fluorodopamine scan after 2 years in 20 patients with PD and 5 years in 11 patients with PD. (Diagnostic criteria not specified)
	Lewitt et al. (2011) (11)	217 (DATATOP)/26	0.89 (0.88)	↓HVA in Selegiline-treated PD patients ($P < 0.001$) (2nd CSF collection)	Well-defined PD patients with long follow-up, 2 CSF samples taken with mean interval of 1.32 years (DATATOP criteria) (98)
Neuromodulin	Sjögren et al. (2000) (37)	23/32	Not specified	↓ ($P < 0.05$)	(Wallin's criteria) (97)
3-hydroxykynurenine	LeWitt et al. (2013) (12)	48/57	14.7 (8.0)	↑ (×1.33) ($P = 0.008671$) ↑3-OH-kynurenine/kynurenine ratio (×1.64) ($P = 0.000835$) (PPV 72% NPV 73%)	CSF collected post-mortem from lateral ventricle (all patients with PD met established neuropathological criteria for PD, Gelb's criteria) (91)
B – Oxidative stress markers					
Advanced Oxidized Protein Products (AOPP self-oxidized)	Garcia-Moreno et al. (2013) (25)	60/45	7.8 (1.2)	↓ lag phase in PD H&Y score 1–2 ($P < 0.05$)	Unspecified number of patients with PD DAT-SPECT-based diagnosis, (diagnostic criteria not specified)
Coeruloplasmin ferroxidase activity	Boll et al. (2008) (22)	22/41	Not specified	↓ ($P = 0.012$)	(Diagnostic criteria not specified)
Oxidized Q10	Isobe et al. (2007) (84)	20/20	2.1 (1.1)	↑oxidized Q10 ($P = 0.0001$) and ↑ [oxidized Q10]/[total Q10] ratio ($P < 0.05$)	Patients with PD untreated (Koller's criteria) (93)
Cu/Zn-superoxide dismutase	Boll et al. (2008) (22)	22/41	Not specified	↓ ($P = 0.021$)	(Diagnostic criteria not specified)
Copper	Boll et al. (2008) (22)	22/41	Not specified	↑ ($P = 0.011$) ROC: spec 84% sens 75% AUC 84%	(Diagnostic criteria not specified)
DJ-1	Hong et al. (2010) (17)	117 (*de novo* 16)/132	8.1 (6.5)	↓ ($P = 5.53$*E-10) spec 50% sens 94%, if ≥65 years spec 90% sens 70%	(UKBBC) (90)
	Herbert et al. (2014) (19)	36/23	3.12 (2.58)	↑ ($P < 0.01$) ROC: spec 53% sens 81% AUC 0.71	3-year follow-up and extended follow-up 3–9 years, (UKBBC) (89)
	Waragai et al. (2006) (18)	40/38	Not specified	↑ ($P < 0.001$) in PD H&Y 1–2	(Calne's criteria) (96)
Glutathione S-transferase Pi	Maarouf et al. (2012) (20)	43/49	Not specified	↓ (×1.6)	CSF collected post-mortem from lateral ventricle (all patients with PD neuropathologically diagnosed, Gelb's criteria) (91)

(continued)

Table 1 *(continued)*

Biomarker candidate	Author (year) (reference number)	Cases/controls	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
Glutathione (oxidized) (GSSG)	LeWitt et al. (2013) (12)	48/57	14.7 (8.0)	↓ (×0.6) ($P = 0.005124$)	CSF collected post-mortem from lateral ventricle (all patients with PD met established neuropathological criteria for PD, Gelb's criteria) (91)
H2O2 and ˙OH	Yu et al. (2014) (27)	62 (36 CI, 3 PDD)/31	Median 2.0, cognitively impaired PD 3.0	↑˙OH ($P = 0.005$) in cognitively impaired PD,←→H2O2	(UKBBC) (89)
Lipid peroxidation	Boll et al. (2008) (22)	22/41	Not specified	↑ ($P = 0.043$)	(Diagnostic criteria not specified)
Nitrites, Nitrates	Boll et al. (2008) (22)	22/41	Not specified	↑ ($P = 0.012$)	(Diagnostic criteria not specified)
Silicic acid (Si)	Forte et al. (2004) (21)	26/13	4.8 (3.8)	↓ ($P < 0.05$)	(London Brain Bank Criteria) (95)
Xanthine	Lewitt et al. (2011) (11)	217 (DATATOP)/26	0.89 (0.88)	↑ [xanthine]/[HVA] ratio (1st CSF collection $P < 0.0016$, 2nd CSF collection $P < 0.001$)	Well-defined PD patients with long follow-up, 2 CSF samples taken with mean interval of 1.32 years (DATATOP criteria) (98)
3-nitrotyrosine products	Fernandez et al. (2013) (26)	54/40	7.8 (1.2)	↑in PD H&Y score I–II ($P < 0.004$)	DAT-SPECT-based diagnosis on all patients with PD (clinical criteria not specified)
8-hydroxyguanosine	Abe et al. (2003) (23)	24/15	2.8 (2.0)	↑ ($P < 0.001$), marked increase	Early untreated PD patients (Koller's criteria) (93)
	Kikuchi et al. (2002) (24)	31/29	male: 6.5 (5.5) female: 4.9 (3.8)	↑ ($P < 0.005$)	(Koller's criteria) (93)
C – Inflammatory and immunological markers					
β2-microglobulin	Zhang et al. (2008) (30)	38/90	Not specified	↑ ($P < 0.001$)	(Gelb's criteria) (91)
IgG	Pisani et al. (2012) (35)	73/47	H&Y 1–2: 2.15 (1.26) H&Y 3–4: 4.5 (3.48)	↑ CSF/serum IgG ratio ($P = 0.002$) in PD H&Y 2.5–4.	MRI performed on all patients with PD (UKBBC) (99)
Interleukins	Yu et al. (2014) (27)	62/31	Median 2.0, cognitively impaired PD 3.0	↑ IL-1-β ($P = 0.000$) and IL-6 ($P = 0.025$) in cognitively impaired PD	(UKBBC) (89)
	Zhang et al. (2008) (30)	38/90	Not specified	↑ IL-8 ($P < 0.01$)	(Gelb's criteria) (91)
IFN-Y	Yu et al. (2014) (27)	62/31	Median 2.0, cognitively impaired PD 3.0	↓ in cognitively impaired PD and non-cognitively impaired PD ($P = 0.03$) ($P = 0.027$)	(UKBBC) (89)
PGE2	Yu et al. (2014) (27)	62/31	Median 2.0, cognitively impaired PD 3.0	←→	(UKBBC) (89)
sCD14	Olsson et al. (2013) (66)	50/37	11.1 (7.4)	←→	(Gelb's criteria) (91)
TNF-α	Yu et al. (2014) (27)	62/31	Median 2.0, cognitively impaired PD 3.0	↓ in cognitively impaired PD ($P = 0.006$)	(UKBBC) (89)
D – Growth factors					
BDNF	Zhang et al. (2008) (30)	38/90	Not specified	↑ ($P < 0.01$)	(Gelb's criteria) (91)
Progranulin	Steinacker (2011) (63)	20/40	Median (range): 6 (0.5–25)	←→	(UKBBC) (89)

(continued)

Table 1 *(continued)*

Biomarker candidate	Author (year) (reference number)	Cases/controls	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
E – Proteins involved in PD pathology					
Amyloids, total tau, phosphorylated tau	Prikrylova Vranová et al. (2010) (59)	32/30	4.4 (3.6)	↑ t-tau ($P = 0.045$)	(UKBBC) (89)
	Trupp et al. (2014) (46)	20/20	Not specified	↓ Aβ1-38 ($P = 0.04$), ↓ Aβ42 ($P = 0.004$) ↓ sAPPα ($P = 0.039$)	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)
	Steinacker et al. (2011) (63)	20/40	Median (range): 6 (0.5–25)	←→ sAPP α+β	(UKBBC) (89)
	Nutu et al. (2013) (62)	90/107	Not specified	↓ Aβ1-15/16 ($P = 0.0005$)	(Gelb's criteria) (91)
	Nielsen et al. (2014) (43)	61 (PDD 26)/51	Not specified	↓ Aβ1-42 ($P < 0.001$), normal p-tau and t-tau	(McKeith's criteria) (92)
	Hall et al. (2012) (48)	90/107	Not specified	↓ t-tau ↓ p-tau ($P < 0.05$).	(Gelb's criteria) (91)
	Wennström et al. (2013) (51)	38/52	Not specified	↓ Aβ1-42 ($P = 0.007$).	(Gelb's criteria) (91)
	Zhang et al. (2008) (30)	38/90	Not specified	↓ t-tau ($P < 0.01$), ↑ Aβ42 ($P < 0.01$) 95% of patients with PD correctly classified as PD in 8 protein multiplex analysis with t-tau as most important driver.	(Gelb's criteria) (91)
	Parnetti et al. (2008) (56)	20/20	Not specified	↓ Aβ42 ($P = 0.03$).	(Gelb's criteria) (91)
	Herbert et al. (2014) (19)	36/23	3.12 (2.58)	←→p-tau ←→ t-tau	3-year follow-up and extended follow-up 3–9 years (UKBBC) (89)
	Süssmuth et al. (2010) (57)	23/20	Median 3.5	←→t-tau	Aβ1-42 and p-tau not measured in controls (UKBBC) (89)
	Sjögren et al. (2000) (37)	23/32	Not specified	←→t-tau ←→ Aβ1-42	(Wallin's criteria) (97)
	Alves et al. (2010) (58)	109 (ParkWest)/36	2.2 (1.7)	↓ Aβ42 ($P = 0.009$) ↓ Aβ40 ($P = 0.008$) ↓ Aβ38 ($P = 0.004$) ←→t-tau ←→ p-tau	Unmedicated newly diagnosed PD patients with average >2-year follow-up (Gelb's criteria) (91)
	Montine et al. (2010) (61)	PD 49/PD-CIND 62/PDD 49/150	PD: 8 (6) PD-CIND: 8 (7) PDD: 16 (6)	↓ p-tau ($P < 0.05$) in PD; ↓Aβ428 ($P < 0.05$) ↓p-tau ($P < 0.01$) in PD-CIND; ↓Aβ42 ($P < 0.01$) in PDD.	(Gibb's criteria) (90)
	Nutu et al. (2013) (64)	43/107	Not specified	←→Aβ1-42	(Gelb's criteria) (91)
	Compta et al. (2012) (60)	33 (PDD 15/33)/12	Median (IQR): PD: 10 (7–15) PDD: 9 (7–10)	↓Aβ1-42 ($P < 0.05$) ↑ t-tau and ↓ Aβ1-42 in PDD ($P < 0.05$)	(UKBBC) (89)
α-synuclein and subspecies	Foulds et al. (2012) (47)	39 (PDD 26/39)/20	14.2 (7.8)	←→:t-α-syn, p-α-syn, o-α-syn	(UKBBC, all patients met neuropathological diagnostic criteria for PD) (89)
	Hong et al. (2010) (17)	117 (*de novo* = 16)/132	8.1 (6.5)	↓ α-syn ($P = 3.85$*E-11) (excluding blood-contaminated samples) sens 93% spec 39%, if 65 years sens 92% spec 58%	(UKBBC) (90)
	Van Dijk et al. (2014) (49)	53/50	6 (5)	↓ α-syn ($P = 0.02$) ↓ α-syn/total protein ratio ($P = 0.00005$)	24 of 53 patients DAT-SPECT-based diagnosis (UKBBC) (89)
	Nielsen et al. (2014) (43)	61 (PDD 26/61)/51	Not specified	←→α-syn	(McKeith's criteria) (92)
	Mondello et al. (2014) (50)	52/22	Median (IQR): 9 (5–17)	↓ α-syn ($P < 0.001$) ROC: sens 89% spec 68% AUC 0.82	(UKBBC) (89)
	Hall et al. (2012) (48)	90/107	Not specified	↓ α-syn ($P < 0.05$),	(Gelb's criteria) (91)
	Aerts et al. (2012) (44)	53/22	3.12 (2.72)	←→α-syn	2-year clinical follow-up on patients, MRI performed on all patients with PD (Gelb's criteria) (91)
	Fernandez et al. (2013) (26)	54/40	7.8 (1.2)	↓α-syn	DAT-SPECT-based diagnosis on all patients with PD (Clinical criteria not specified)

(continued)

Table 1 *(continued)*

Biomarker candidate	Author (year) (reference number)	Cases/controls	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
	Tokuda et al. (2010) (45)	1. Main study: 28/28 2. Cross-sectional pilot study: 25/43	04:12	1. ←→α-syn, ↑ o-α-syn ($P < 0.05$) ROC: sens 75% spec 87.5% AUC 0.859 ↑ o-α-syn/t-α-syn ratio ($P < 0.001$) ROC: sens 89.3 spec 90.6 AUC 0.948, ratio highest in early or mild PD 2. ↑ o-α-syn ($P < 0.05$)	(UKBBC) (90)
	Herbert et al. (2014) (19)	43/30	3.12 (2.58)	←→α-syn	3-year follow-up and extended follow-up 3–9 years (UKBBC) (89)
	Trupp et al. (2014) (46)	20/20	Not specified	←→α-syn	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)
	Wennström et al. (2013) (51)	38/52	Not specified	↓α-syn ($P = 0.003$)	(Gelb's criteria) (91)
	Shi et al. (2014) (86)	100/100	7.7 (5.9)	↓α-syn ($P < 0.0001$) ROC: sens 76.8% spec 53.5% AUC 0.724	(UKBBC, not excluding PD patients with >1 relative affected by PD) (90)
Neurosin	Nielsen et al. (2014) (43)	61 (PDD 26/61)/51	Not specified	←→	(McKeith's criteria) (92)
	Wennström et al. (2013) (51)	38/52	Not specified	↓ ($P = 0.006$)	(Gelb's criteria) (91)
GFAP	Süssmuth et al. (2010) (57)	23/20	Median 3.5	←→	(UKBBC) (89)
Clusterin	Maarouf et al. (2012) (20)	43/49	Not specified	↑ (×2)	CSF collected post-mortem from lateral ventricle (all patients with PD neuropathologically diagnosed, Gelb's criteria) (91)
	Prikrylova Vranová et al. (2010) (59)	32/30	4.4 (3.6)	↑ ($P = 0.004$)	(UKBBC) (89)
Neurofilament Light Chain (NfL)	Hall et al. (2012) (48)	90/107	Not specified	←→	(Gelb's criteria) (91)
	Trupp et al. (2014) (46)	20/20	Not specified	←→	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)
Neurofilament Heavy Chain (NfH)	Brettschneider et al. (2006) (70)	22/45	Median (range): 4 (0.3–2.5)	←→	(UKBBC) (89)
	Steinacker et al. (2011) (63)	20/40	Median (range): 6 (0.5–25)	←→	(UKBBC) (89)
YKL-40	Olsson et al. (2013) (66)	50/37	H&Y 1–2: 2.15 (1.26) H&Y 3–4: 4.5 (3.48)	↓ ($P < 0.05$) sens 60.5% spec 81.5%	(Gelb's criteria) (91)
Neuron Glia 2	Nielsen et al. (2014) (43)	61 (PDD 26/61)/51	Not specified	←→	(McKeith's criteria) (92)
Ubiquitin	Oeckl et al. (2014) (85)	25/28	Not specified	←→	(McKeith's criteria) (92)
UCHL-1	Mondello et al. (2014) (50)	52/22	Median (IQR): 9 (5–17)	↓ ($P < 0.001$) ROC: sensitivity 87% specificity 79% AUC 0.82	(UKBBC) (89)
Apolipoprotein A1 and A2	Zhang et al. (2008) (30)	38/90	Not specified	←→apolipoprotein A1 ↓ apolipoprotein A2 ($P < 0.01$)	(Gelb's criteria) (91)
	Maarouf et al. (2012) (20)	43/49	Not specified	↓ apolipoprotein A1 in PD (×1.6)	CSF collected post-mortem from lateral ventricle (all patients with PD neuropathologically diagnosed, Gelb's criteria) (91)
Apolipoprotein ε	Maarouf et al. (2012) (20)	43/49	Not specified	↑ (×2)	CSF collected post-mortem from lateral ventricle (all patients with PD neuropathologically diagnosed, Gelb's criteria) (91)
	Alves et al. (2010) (58)	109 (ParkWest)/36	2.2 (1.7)	←→	Unmedicated newly diagnosed PD patients with average >2-year follow-up (Gelb's criteria) (91)

(continued)

Table 1 *(continued)*

Biomarker candidate	Author (year) (reference number)	Cases/controls	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
	Zhang et al. (2008) (30)	38/90	Not specified	↓ ($P < 0.01$)	(Gelb's criteria) (91)
Transthyretin	Maarouf et al. (2012) (20)	43/49	Not specified	↑ (×2.7)	CSF collected post-mortem from lateral ventricle (all patients with PD neuropathologically diagnosed, Gelb's criteria) (91)
F – Other					
Albumin	Pisani et al. (2012) (35)	73/47	H&Y 1–2: 2.15 (1.26) H&Y 3–4: 4.5 (3.48)	↑ CSF-/serum albumin ratio ($P = 0.02$)	MRI performed on all patients with PD (UKBBC) (99)
Corticosterone	LeWitt et al. (2013) (12)	48/57	14.7 (8.0)	↓ (×0.72) ($P = 0.009262$)	CSF collected post-mortem from lateral ventricle (all patients with PD met established neuropathological criteria for PD, Gelb's criteria) (91)
Creatinine	Trupp et al. (2014) (46)	20/20	Not specified	↓ ($P = 0.007$)	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)
Fibrinogen	Maarouf et al. (2012) (20)	43/49	Not specified	↓ (×4.34)	CSF collected post-mortem from lateral ventricle (all patients with PD neuropathologically diagnosed, Gelb's criteria) (91)
Haptoglobin	Zhang et al. (2008) (30)	38/90	Not specified	←→	(Gelb's criteria) (91)
Insulin	Jimenez-Jimenez et al. (2000) (100)	24/15	4.9 (5.1)	←→	(Hughes' criteria) (89)
Vitamin D-binding protein	Zhang et al. (2008) (30)	38/90	Not specified	↑ ($P < 0.01$)	(Gelb's criteria) (91)
Xylitol	Trupp et al. (2014) (46)	20/20	Not specified	↓ ($P = 0.017$)	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)
3-hydroxyisovaleric acid	Trupp et al. (2014) (46)	20/20	Not specified	↓ ($P = 0.002$)	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)
Tryptophan	Trupp et al. (2014) (46)	20/20	Not specified	↓ ($P = 0.003$)	DAT-SPECT abnormal in all patients with PD and in no controls, 3-year follow-up (UKBBC)

↑ = increase ↓ = decrease ←→ = no difference; changes in biomarkers are described as changes in patients with Parkinson's disease vs controls. Disease duration is recorded when collecting cerebrospinal fluid. It is defined as years since onset of Parkinson's disease symptoms and shown as mean (± standard deviation) unless otherwise specified.

L-DOPA, L-3;4-dihydroxyphenylalanine; UKBBC, United Kingdom Brain Bank Criteria; DA, dopamine; DHPG, dihydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HIAA, 5-hydroxyindoleacetic acid; NA, noradrenalin; PD, Parkinson's disease; sens, sensitivity; spec, specificity; DATATOP, Deprenyl and Tocopherol Antioxidative Therapy Of Parkinsonism; CSF, Cerebrospinal fluid; H&Y score, Hoehn & Yahr score; DAT-SPECT, dopamine transporter single-photon emission computed tomography; Q10, Coenzyme Q10; Cu, copper; Zn, Zinc; ROC, receiver operation characteristics; AUC, area under the curve; H202, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; CI, cognitively impaired; PDD, Parkinson's disease dementia; IgG, immunoglobulin G; MRI, magnetic resonance imaging; IFN-Y, interferon-gamma; PGE2, prostaglandin E2; sCD14, soluble cluster of differentiation 14; TNF-α, tumour necrosis factor-α; BDNF, brain-derived neurotrophic factor; t-tau, total tau; Aβ, amyloid-β; sAPPα, soluble amyloid precursor protein-α; NINDS, National Institute of Neurological Disorders and Stroke; p-tau, phosphorylated tau; ParkWest, Norwegian population-based study of the incidence; neurobiology; and prognosis of PD; CIND, cognitively impaired – non-demented; IQR, interquartile range; t-α-syn, total α-synuclein; p-α-syn, phosphorylated α-synuclein; o-α-syn, oligomeric α-synuclein; GFAP, glial fibrillary acidic protein; YKL-40, human cartilage glycoprotein-39; UCHL-1, ubiquitin carboxy-terminal hydrolase L1.

nervous system (CNS). Forte et al. (21) found a significantly lower mean concentration of Si in CSF of patients with PD, although the mean concentration of Al in CSF was not significantly different.

In 2008, Boll et al. (22) examined several aspects of oxidative stress showing a significant increase in copper (Cu) in CSF of patients with PD, along with a decrease in Cu/Zn-superoxide dismutase activity and ceruloplasmin ferroxidase activity which plays a role in iron homeostasis. Boll et al. also found increased markers of lipoprotein peroxidation in CSF from patients with PD.

Abe et al. (23) and Kikuchi et al. (24) found a significant increase in 8-hydroxyguanosine (8-OHG), a marker of hydroxyl radical damage of ribonucleic acid (RNA), in PD CSF as compared to controls.

PD vs other neurodegenerative disorders – DJ-1 was found by Hong et al. (17) to be significantly lower in PD compared with Alzheimer's disease (AD), whereas Herbert et al. (19) found DJ-1 to be significantly lower in PD compared with MSA, and combined with total tau-protein (t-tau) and phosphorylated tau-protein (p-tau) distinguished PD from MSA with a sensitivity of 82% and specificity of 81% (see Table 2).

Disease severity and cognitive abilities – Garcia-Moreno et al. (25) showed, through enzyme-linked immune assays (ELISA) that the amount of advanced oxidized protein products (AOPP, a marker for protein halogenation which is the result of free radicals) in CSF increased significantly faster in incubated CSF from PD patients with Hoehn & Yahr (H&Y) score 1–2 compared with H&Y score 3–4 and controls, maybe indicating a susceptibility to oxidative damage in early disease stages. Corroborating this notion, Fernandez et al. (26) showed an increase in unspecific 3-nitrotyrosine protein products (nitrosylation of proteins being an oxidative damage mechanism) in PD patients with H&Y score 1–2 compared with advanced PD and controls. Under the notion of a more pronounced oxidative damage occurring in early PD, DJ-1 was also found by Herbert et al. (19) to be significantly more increased in PD patients with H&Y score 1–2 compared with H&Y score 3–4. 8-OHG was found to be negatively correlated to disease duration (with no correlation to age) by Abe et al. (23), which might indicate lesser oxidative stress in advanced disease stages.

Boll et al. (22) found Cu significantly positively correlated to both UPDRS part III motor score and disease duration and found the ceruloplasmin ferroxidase activity to be related to the time of onset (understood by the authors as disease duration).

Related to cognition, Yu et al. (27) through chemical colorimetric analysis showed that the hydroxyl radical $^{\cdot}OH$ was negatively correlated to the MOCA score (MOntreal Cognitive Assessment) and significantly increased in CSF of cognitively impaired PD patients compared with healthy controls. See Table 3 for an overview.

Prognostic value – In a large and well-defined group of patients with PD participating in the DATATOP trial (Deprenyl and Tocopherol Antioxidative Therapy Of Parkinsonism), Ascherio et al. (28) showed that CSF levels of the antioxidant uric acid, the end product of the purine metabolism, were negatively correlated with the severity of disease as assessed by the need for L-DOPA therapy and clinical scores using the UPDRS.

Inflammatory and immunological markers

Microglial cells are active in the substantia nigra of patients with PD, and a secondary dysregulated inflammatory response (e.g. a deregulation of the interplay between glucocorticoids and glucocorticoid receptors in dopaminergic neurons) seems to play a role in the progression of the disease, maybe triggered by the primary disease mechanism (29).

PD vs controls – Using a multiplex assay for cytokines, Zhang et al. (30) found the levels of eight cytokines altered in PD as compared to controls. Among these were higher concentrations of β2-microglobulin, a component of a ubiquitous cell surface protein, used in staging of multiple myeloma and proposed to have an immunological function (31). They also found an increase in IL-8 (interleukin-8), whereas Yu et al. (27) found an increase in IL-6 and IL-1-β in cognitively impaired PD patients compared with controls. Yu et al. found that concentrations of the neuroinflammatory factors interferon-gamma (IFN-Y) and tumour necrosis factor-alpha (TNF-α) were also increased in PD, although the latter only in cognitively impaired patients.

PD vs other neurodegenerative diseases – Constantinescu et al. (32) showed that patients with atypical PD had higher concentrations of β2-microglobulin than patients with PD and controls, only reaching significance when combined

Table 2 An overview of cerebrospinal fluid biomarker candidates differentiating Parkinson's disease from other neurodegenerative diseases

Biomarker candidate	Author (year) (reference number)	Patients with PD/Other neurodegenerative diseases	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
A – Neurotransmitters and neuromodulators					
DA, DHPG, DOPA, DOPAC, HVA, MHPG, 5-HIAA and NA	Goldstein et al. (2012) (9)	PD 34/MSA 54/PAF 20	6.0 (1)	↑DHBG vs PAF ($P = 0.001$)	DOPA washout on all patients with PD to avoid effect of L-DOPA-treatment on measurements (Diagnostic criteria not specified)
	Goldstein et al. (2008) (10)	PD 77/MSA 57	9.0 (1)	DOPAC←→ DA←→	Follow-up 6-[18F]-fluorodopamine scan after 2 years in 20 patients with PD and 5 years in 11 patients with PD.
	Abdo et al. (2007) (13)	PD 31/MSA-P 19	3.6 (2.8)	↑ MHPG, HVA, 5-HIAA vs MSA-P ($P > 0.0001$) ROC (MHPG): sens 94% spec 83% AUC 0.91	Minimum 2-year clinical follow-up to confirm diagnosis (UKBBC)
Neuromodulin	Sjögren et al. (2000) (37)	PD 23/AD 60	Not specified	↓ vs AD ($P < 0.001$, $P < 0.05$)	(Wallin's criteria) (97)
Orexin	Yasui et al. (2006) (87)	PD 62/DLB 13/PSP 16/CBD 7	6.0 (4.9)	↑ vs CBD ($P < 0.05$). ↓ vs PSP ($P < 0.001$)	(Calne's criteria) (96)
B – Oxidative stress markers					
Coeruloplasmin ferroxidase activity	Boll et al. (2008) (22)	PD 22/HD 23/ALS 27/pAD 8	Not specified	←→	(Diagnostic criteria not specified)
Cu/Zn-superoxide dismutase	Boll et al. (2008) (22)	PD 22/HD 23/ALS 27/pAD 8	Not specified	←→	(Diagnostic criteria not specified)
Copper	Boll et al. (2008) (22)	PD 22/HD 23/ALS 27/pAD 8	Not specified	←→	(Diagnostic criteria not specified)
DJ-1	Hong et al. (2010) (17)	PD 117 (*de novo* $n = 16$)/AD 50	8.1 (6.5)	↓ vs AD ($P = 0.00016$)	(UKBBC) (90)
	Herbert et al. (2014) (19)	PD 43/MSA 23	3.12 (2.58)	↓ vs MSA ($P < 0.001$)	3-year follow-up and extended follow-up 3–9 years (UKBBC) (89)
Lipid peroxidation	Boll et al. (2008) (22)	PD 22/HD 23/ALS 27/pAD 8	Not specified	←→	(Diagnostic criteria not specified)
Nitrites, Nitrates	Boll et al. (2008) (22)	PD 22/HD 23/ALS 27/pAD 8	Not specified	←→	(Diagnostic criteria not specified)
8-hydroxyguanosine	Kikuchi et al. (2002) (24)	PD 31/probable MSA 16	Male: 6.5 (5.5) Female: 4.9 (3.8)	↓ vs MSA ($P < 0.05$)	(Koller's criteria) (93)
C – Inflammatory and immunological markers					
β2-microglobulin	Constantinescu et al. (2010) (32)	PD 56/MSA 42/PSP 39/CBD 9	11:00	↓ vs PSP ($P = 0.0005$) Differs PD from APD in proteomic profiling with ubiquitin and chromogranin fragments 1 and 2 (AUC 0.80)	(UKBBC definite PD) (89)
	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)
Flt3-ligand	Siljadzic et al. (2014) (33)	PD 52/MSA 34/PSP 32	10.1 (6.2)	←→	(Gelb's criteria) (91)
Interleukins	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)
sCD14	Olsson et al. (2013) (66)	PD 50/PSP 32/CBD 10/MSA 37	11.1 (7.4)	←→	(Gelb's criteria) (91)
D – Growth factors					
BDNF	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)

(continued)

Table 2 *(continued)*

Biomarker candidate	Author (year) (reference number)	Patients with PD/Other neurodegenerative diseases	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
E – Proteins involved in PD pathology					
Amyloids, total tau, phosphorylated tau	Bech et al. (2012) (67)	PD 22/MSA 10/PSP 10/DLB 11	Median (range): 7 (*2–22*)	↑ Aβ1-42 vs DLB ($P = 0.002$) sens 82% spec 79% ↑ APPβ ($P = 0.02$) ↑ APPα ($P = 0.03$) vs DLB and MSA. [p-tau]/[t-tau] ratio: ←→	MRI on all patients, median 16-month follow-up to confirm diagnosis (UKBBC definite PD)
	Abdo et al. (2007) (13)	PD 31/MSA-P 19	3.6 (2.8)	↓ t-tau vs MSA-P ($P < 0.0001$ ROC: sens 76% spec 97% AUC 0.88	Minimum 2-year clinical follow-up to confirm diagnosis (UKBBC)
	Hall et al. (2012) (48)	PD 90/AD 48/MSA 48/CBD 12/PSP 45/DLB 70/PDD 33	Not specified	↑ Aβ1-42↓ p-tau ↓ t-tau vs AD ($P < 0.001$) 5 biomarker combination can distinguish between PD and APD with sensitivity of 85%, specificity of 92%, AUC 0.93.	(Gelb's criteria) (91)
	Wennström et al. (2013) (51)	PD 38/PDD 22/DLB 33/AD 46	Not specified	↓ t-tau and p-tau vs AD ($P < 0.001$)	(Gelb's criteria) (91)
	Mollenhauer et al. (2011) (68)	1. cohort: PD 51/AD 62/DLB 55/MSA 29/Neurological controls 76 2. cohort: PD 273/NPH 22/PSP 8/MSA 15/Neurological controls 23	1. cohort: 12.17 (6.42) 2. cohort: 6.17 (3.5)	Differentiation of synucleinopathies from non-synucleinopathies with age, tau and α-syn (AUC in 1. cohort 0.908 and 0.729 in 2. cohort)	(Gibbs' criteria) (90)
	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	↑ Aβ42 and ↓ t-tau vs AD.	(Gelb's criteria) (91)
	Parnetti et al. (2008) (56)	PD 20/PDD 18/AD 23/DLB 19	Not specified	↓ t-tau vs DLB, PDD and AD ($P = 0.01$, $P = 0.03$, $P = 0.000$). ↓ P-tau vs AD	(Gelb's criteria) (91)
	Herbert et al. (2014) (19)	PD 43/MSA 23	3.12 (2.58)	↓t-tau vs MSA ($P < 0.01$)	3-year follow-up and extended follow-up 3–9 years (UKBBC) (89)
	Süssmuth et al. (2010) (57)	PD 23/PSP 27/PSP-P 7/RS 20/MSA-P 11/MSA-C 14	Median 3.5	↓ t-tau vs PSP-P, MSA-P and MSA-C ($P = 0.001$, $P = 0.028$, $P = 0.028$). ↑ [p-tau]/[t-tau] ratio vs RS, PSP-P, MSA ($P = 0.024$, $P = 0.016$, $P = 0.002$, $P = 0.001$)	(UKBBC) (89)
	Sjögren et al. (2000) (37)	PD 23/FTD 17/AD 60	Not specified	↓ t-tau ($P < 0.001$) and ↑ Aβ1-42 ($P < 0.001$) vs AD.	(Wallin's criteria) (97)
	Alves et al. (2010) (58)	PD 109 (ParkWest)/AD 20	2.2 (1.7)	↑Aβ ($P = 0.002$), ↓ t-tau and p-tau ($P < 0.001$, $P < 0.002$) in PD compared with AD.	Unmedicated newly diagnosed PD patients with average >2-year follow-up (Gelb's criteria) (91)
	Montine et al. (2010) (61)	PD 49/PD-CIND 62/PDD 49/AD 49/aMCI 24	PD: 8 (6) PD-CIND: 8 (7) PDD: 16 (6)	↓ amount of patients with abnormal p-tau/Aβ1-42 ratio vs AD	(Gibbs' criteria) (90)
	Nutu et al. (2013) (64)	PD 43/PDD 33/DLB 51/AD 48		↑ Aβ1-42 ($P < 0.001$) ↑ Aβ1-42/Aβ40 ratio ($P < 0.001$) vs AD.	(Gelb's criteria) (91)

(continued)

Table 2 *(continued)*

Biomarker candidate	Author (year) (reference number)	Patients with PD/Other neurodegenerative diseases	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
α-synuclein and subspecies	Foulds et al. (2012) (47)	PD 39 (PDD 26/39)/DLB 17/PSP 12/MSA 8	14.2 (7.8)	↑ phosphorylated oligomeric α-syn in MSA vs PD, PDD, DLB, PSP.	(UKBBC, all patients met neuropathological diagnostic criteria for PD) (89)
	Mollenhauer et al. (2011) (68)	1.cohort:PD 51/AD 62/DLB 55/MSA 29/Neurological controls 76 2. cohort: PD 273/NPH 22/PSP 8/MSA 15/Neurological controls 23	1. cohort: 12.17 (6.42) 2. cohort: 6.17 (3.5)	↓ t-α-syn vs AD ($P = 0.0002$) α-syn predictor of synucleinopathy ($P < 0.0001$)	(Gibbs' criteria) (90)
	Nielsen et al. (2014) (43)	PD 35/PDD 26/DLB 37	Not specified	←→t-α-syn	(NINDS)
	Hall et al. (2012) (48)	PD 90/AD 48/MSA 48/CBD 12/PSP 45/DLB 70/PDD 33	Not specified	↓t-α-syn vs AD ($P < 0.001$) and PSP ($P < 0.05$)	(Gelb's criteria) (91)
	Aerts et al. (2012) (44)	PD 53/MSA 47/DLB 3/vascular PD 22	3.12 (2.72)	←→t-α-syn	2-year clinical follow-up on patients, MRI performed on all patients with PD (Gelb's criteria) (91)
	Mondello et al. (2014) (50)	PD 52/MSA 34/PSP 32/CBD 12	Median (IQR): 9 (5–17)	↓t-α-syn in synucleinopathies vs tauopathies ($P < 0.015$)	(UKBBC) (89)
	Tokuda et al. (2010) (45)	Cross-sectional pilot study: PD 25/AD 35/PSP 18	04:12	↑ o-α-syn vs PSP ($P < 0.05$) and AD ($P < 0.001$)	(UKBBC) (90)
	Herbert et al. (2014) (19)	PD 43/MSA 23	3.12 (2.58)	←→t-α-syn	3-year follow-up and extended follow-up 3–9 years (UKBBC) (89)
	Wennström et al. (2013) (51)	PD 38/PDD 22/DLB 33/AD 46	Not specified	↓t-α-syn vs AD ($P = 0.003$)	(Gelb's criteria) (91)
Neurosin	Nielsen et al. (2014) (43)	PD 35/PDD 26/DLB 37	Not specified	←→	(NINDS)
	Wennström et al. (2013) (51)	PD 38/PDD 22/DLB 33/AD 46	Not specified	↓ in vs AD	(Gelb's criteria) (91)
GFAP	Süssmuth et al. (2010) (57)	PD 23/PSP 27/PSP-P 7/RS 20/MSA-P 11/MSA-C 14	Median 3.5	←→	(UKBBC) (89)
Neurofilament Light Chain (NfL)	Abdo et al. (2007) (13)	PD 31/MSA-P 19	3.6 (2.8)	↓ vs MSA ($P < 0.0001$) ROC: sens 83% spec 90% AUC 0.92	Minimum 2-year clinical follow-up to confirm diagnosis (UKBBC)
	Bech et al. (2012) (67)	PD 22/MSA 10/PSP 10/DLB 11	Median (range): 7 (*2–22*)	↓ vs MSA, PSP and DLB ($P < 0.0001$) sens 86% spec 81%	MRI on all patients, median 16-month follow-up to confirm diagnosis (UKBBC definite PD)
	Hall et al. (2012) (48)	PD 90/AD 48/MSA 48/CBD 12/PSP 45/DLB 70/PDD 33	Not specified	↓ vs AD ($P < 0.001$) ↓ vs APD ($P < 0.001$) 5 biomarker combination can distinguish between PD and APD with sens 85%, spec 92% AUC 0.93	(Gelb's criteria) (91)
Neurofilament Heavy Chain (NfH)	Brettschneider et al. (2006) (70)	PD 22/PSP 21/CBD 6/MSA 21	Median (range): 4 (0.3–2.5)	↓ vs PSP ($P < 0.05$) sens 76.5% spec 94.4%	Considerable overlap between PSP and PD (UKBBC) (89)
	Abdo et al. (2007) (13)	PD 31/MSA-P 19	3.6 (2.8)	↓vs MSA (<0.0001) ROC: sens 83% spec 87% AUC 0.88	Minimum 2-year clinical follow-up to confirm diagnosis (UKBBC)
YKL-40	Olsson et al. (2013) (66)	PD 50/PSP 32/CBD 10/MSA 37	11.1 (7.4)	↓ vs PSP, CBD ($P < 0.0001$) and MSA ($P < 0.001$)	(Gelb's criteria) (91)
Neuron Glia 2	Nielsen et al. (2014) (43)	PD 35/PDD 26/DLB 37	Not specified	←→	(NINDS)

(continued)

Table 2 *(continued)*

Biomarker candidate	Author (year) (reference number)	Patients with PD/Other neurodegenerative diseases	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
Ubiquitin	Constantinescu et al. (2010) (32)	PD 56/MSA 42/PSP 39/CBD 9	11:00	↓ vs PSP ($P = 0.002$) Differs PD from APD in proteomic profiling with β2-microglobulin and chromogranin fragments 1 and 2 (AUC 0.80)	(UKBBC definite PD) (89)
	Oeckl et al. (2014) (85)	PD 25/ALS 20/PSP 17/bFTD 15/totFTD 28/PPA 6/CJD 19	Not specified	↓ vs CJD and AD	(McKeith's criteria) (92)
UCHL-1	Mondello et al. (2014) (50)	PD 52/MSA 34/PSP 32/CBD 12	Median (IQR): 9 (5–17)	↓ vs MSA, PSP and CBD ($P = 0.0003$) ROC (PD vs APD): sensitivity 58% specificity 77% AUC 0.69	(UKBBC) (89)
Apolipoprotein A1 and A2	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)
Apolipoprotein ε	Alves et al. (2010) (58)	PD 109 (ParkWest)/AD 20	2.2 (1.7)	↓ vs AD ($P < 0.001$)	Unmedicated newly diagnosed PD patients with average >2-year follow-up (Gelb's criteria) (91)
	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)
F – Other					
Haptoglobin	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)
Vitamin D-binding protein	Zhang et al. (2008) (30)	PD 38/AD 38	Not specified	←→	(Gelb's criteria) (91)

↑ = increase ↓ = decrease ←→ = no difference; changes in biomarkers are described as changes in patients with Parkinson's disease vs other neurodegenerative diseases unless otherwise specified. Disease duration is recorded when collecting cerebrospinal fluid. It is defined as years since onset of Parkinson's disease symptoms and is shown as mean (± standard deviation) unless otherwise specified. Disease duration is only described for patients with PD. Diagnostic criteria are only specified for patients with Parkinson's disease. DA, dopamine; DHPG, dihydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; L-DOPA, L-3;4-dihydroxyphenylalanine; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HIAA, 5-hydroxyindoleacetic acid; NA, noradrenalin; PD, Parkinson's disease; MSA, multiple system atrophy; PAF, pure autonomic failure; ROC, receiver operating characteristics; sens, sensitivity; spec, specificity; AUC, area under the curve; DLB, Lewy body dementia; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; UKBBC, United Kingdom Brain Bank Criteria; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; pAD, probable Alzheimer's disease; Cu, copper; Zn, zinc; AD, Alzheimer's disease; APD, atypical parkinsonian disorder; Flt3-ligand, Fms-related tyrosine kinase 3 ligand; sCD14, soluble cluster of differentiation 14; BDNF, brain-derived neurotrophic factor; Aβ, amyloid-β; p-tau, phosphorylated tau; t-tau, total tau; APP, amyloid precursor protein; MRI, magnetic resonance imaging; MSA-P, multiple system atrophy with predominant parkinsonism; NPH, normal pressure hydrocephalus; PSP-P, progressive supranuclear palsy with predominant parkinsonism; RS, Richardson's syndrome; MSA-C, multiple system atrophy with predominant cerebellar ataxia; FTD, frontotemporal dementia; PDD, Parkinson's disease dementia; α-syn, α-synuclein; PD-CIND, Parkinson's disease; cognitively impaired non-demented; aMCI, amnestic mild cognitive impairment; t-α-syn, total α-synuclein; NINDS, National Institute of Neurological Disorders and Stroke; IQR; interquartile range; o-α-syn, oligomeric α-synuclein; GFAP, glial fibrillary acidic protein; YKL-40, human cartilage glycoprotein-39; bFTD, behavioural variant frontotemporal dementia; totFTD, total frontotemporal dementia; PPA, primary progressive aphasia; CJD, Creutzfeldt–Jakob's disease; UCHL-1, ubiquitin carboxy-terminal hydrolase L1.

Table 3 An overview of cerebrospinal fluid biomarker candidates related to cognitive abilities, disease severity and prognostic biomarker candidates related to cognition and disease severity

Biomarker candidate	Author (year) (reference number)	Patients (*n*)	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
A – Neurotransmitter and neuromodulators					
DA, DHPG, DOPA, DOPAC, HVA, MHPG, 5-HIAA and NA	Lewitt et al. (2011) (11)	217 (DATATOP)	0.89 (0.88)	[xanthine]/[HVA] ratio negatively correlates with UPDRS part 3 motor score ↓ [xanthine]/[HVA] ratio in UPDRS 2 + 3 score ≥40 vs <20 ($P = 0.021$)	Well-defined PD patients with long follow-up (DATATOP criteria) (98)
	Goldstein et al. (2012) (9)	34	6.0 (1.0)	↑DHPG vs PD patients with orthostatic hypotension ($P = 0.002$)	
B – Oxidative stress markers					
Advanced Oxidized Protein Products (AOPP self-oxidized)	Garcia-Moreno et al. (2013) (25)	60	7.8 (1.2)	↓ lag phase in PD H&Y score 1–2 vs PD H&Y score 3–4 ($P < 0.05$)	Unspecified number of patients with PD DAT-SPECT-based diagnosis, (diagnostic criteria not specified)
Coeruloplasmin ferroxidase activity	Boll et al. (2008) (22)	22	Not specified	Positively correlated with time of onset of PD ($P < 0.007$)	(Diagnostic criteria not specified)
Oxidized Q10	Isobe et al. (2007) (84)	20	2.1 (1.1)	[oxidized Q10]/[total Q10] ratio negatively correlated to disease duration ($P < 0.01$)	PD patients untreated (Koller's criteria) (94)
Copper	Boll et al. (2008) (22)	22	Not specified	Significant correlation with disease duration ($P = 0.05$) and UPDRS part 3 motor score ($P < 0.05$)	(Diagnostic criteria not specified)
DJ-1	Waragai et al. (2006) (18)	40	Not specified	↑ in PD H&Y score 1–2 vs PD H&Y score 3–4	(Calne's criteria) (96)
Lipid peroxidation	Boll et al. (2008) (22)	22	Not specified	Positive correlation with time of disease onset.	(Diagnostic criteria not specified)
Urate	Ascherio et al. (2009) (28)	800 (DATATOP)	<5 years	↑ urate correlated with↓ UPDRS-change rate ($P = 0.05$), ↑ urate decreased necessity of commencing L-DOPA-treatment	Well-defined PD patients, 24-month follow-up (DATATOP criteria) (98)
Xanthine	Lewitt et al. (2011) (11)	217 (DATATOP)	0.89 (0.88)	[Xanthine]/[HVA] ratio correlates with severity of UPDRS part 3 motor score	Well-defined PD patients with long follow-up, 2 CSF samples taken with mean interval of 1.32 years (DATATOP criteria) (98)
3-nitrotyrosine products	Fernandez et al. (2013) (26)	54	7.8 (1.2)	↑ 3-nitrotyrosine products in PD H&Y score 1–2 vs advanced PD	DAT-SPECT based diagnosis on all patients with PD (Clinical criteria not specified)
8-hydroxyguanosine	Abe et al. (2003) (23)	24	2.8 (2.0)	Negatively correlated with disease duration ($P < 0.05$)	Early untreated PD patients (Koller's criteria) (93)
C – Inflammatory and immunological markers					
β2-microglobulin	Leverenz et al. (2011) (72)	22	Not specified	No correlation to cognitive performance	(UKBBC) (90)
Flt3-ligand	Silajdzic et al. (2014) (33)	52	10.1 (6.2)	Positive correlation with H&Y score.	(Gelb's criteria) (91)
IgG	Pisani et al. (2012) (35)	73	H&Y 1–2: 2.15 (1.26) H&Y 3–4: 4.5 (3.48)	↑ CSF/serum IgG ratio ($P = 0.002$) in PD H&Y 2.4-4, ratio positively correlated with H&Y score.	MRI performed on all patients with PD (UKBBC) (99)
Interleukins	Yu et al. (2014) (27)	62	Median 2.0, cognitively impaired PD 3.0	↑ IL-1-β ($P = 0.000$) ↑ IL-6 ($P = 0.025$) in cognitively impaired PD	(UKBBC) (89)
	Leverenz et al. (2011) (72)	22	Not specified	No correlation between IL-8 and cognitive performance	(UKBBC) (90)
Serpin A1 (α1-antitrypsin)	Jesse et al. (2012) (88)	PD 24/PDD 21	Not specified	↑isoforms of serpin A1 in PDD ($P = 0.02$) sens 100% spec 58%	(McKeith's criteria) (92)

(continued)

Table 3 *(continued)*

Biomarker candidate	Author (year) (reference number)	Patients (*n*)	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
D – Growth factors					
BDNF	Leverenz et al. (2011) (72)	22	Not specified	No relation to cognitive performance	(UKBBC) (90)
E – Proteins involved in PD pathology					
Amyloids, total tau, phosphorylated tau	Prikrylova Vranová et al. (2010) (59)	32/30	4.4. (3.6)	PD diagnosis > 2 years: ↑t-tau ($P = 0.033$) ↑ t-tau/Aβ ratio ($P = 0.011$)	(UKBBC) (89)
	Parnetti et al. (2008) (56)	PD 20/PDD 18	Not specified	↓ t-tau vs PDD ($P = 0.03$).	(Gelb's criteria) (91)
	Alves et al. (2010) (58)	109 (ParkWest)	2.2 (1.7)	Significant correlation between Aβ-types and memory impairment Aβ42 ($P = 0.029$) Aβ40 ($P < 0.001$) Aβ38 ($P = 0.001$)	Unmedicated newly diagnosed PD patients with average >2-year follow-up (Gelb's criteria) (91)
	Montine et al. (2010) (61)	PD 49/PD-CIND 62/PDD 49	PD: 8 (6) PD-CIND: 8 (7) PDD: 16 (6)	Aβ1-42: PD > PD-CIND > PDD.	(Gibb's criteria) (90)
	Nutu et al. (2013) (64)	PD 43/PDD 33	Not specified	Aβ1-40 and Aβ1-42 positively correlated to MMSE ($P = 0.015$, $P < 0.01$).	(Gelb's criteria) (91)
	Compta et al. (2012) (60)	PD 18/PDD 15	Median (IQR): PD: 10 (7–15) PDD: 9 (7–10)	All *P*-values are $P < 0.05$. ↓t-tau, ↓p-tau and↑ Aβ1-42 in PDND compared with PDD. Negative correlation between t-tau, p-tau and MRI-measured GMV in specific cortical areas. Positive correlation between Aβ1-42 and GMV in specific cortical areas. T-tau negatively correlated with memory, naming and visuospatial abilities. Aβ1-42 positively correlated with semantic and phonetic fluency, naming and visuospatial score.	(UKBBC) (89)
	Zhang et al. (2013) (73)	403 (DATATOP)	2.04 (at baseline)	↓ baseline Aβ1-42 correlated with ↓ baseline UPDRS ($P = 0.035$) Baseline p-tau/t-tau ratio negatively correlated with change rate in UPDRS ($P = 0.006$)+UPDRS motor score ($P = 0.002$). Baseline p-tau/AB1-42 negatively correlated with change rate of total UPDRS ($P = 0.001$) and motor UPDRS ($P = 0.005$). Change rate of t-tau positively correlated with change rate of: total UPDRS ($P = 0.0005$), motor UPDRS ($P = 0.002$) Change rate t-tau/AB1-42 positively correlated with change rate of total UPDRS ($P = 0.007$), motor UPDRS ($P = 0.006$)	Well-defined PD patients with long follow-up (DATATOP criteria) (98)
	Liu et al. (2015) (74)	(DATATOP) Discovery cohort: Phase 1: 201 Phase 2: 154 Validation cohort: Phase 1: 202 Phase 2: 151	Phase 1: Discovery: 1.99 Validation: 2.08 Phase 2: Discovery: 3.82 Validation: 3.83	Aβ1-42 not correlated with cognitive decline. T-tau and p-tau/Aβ1-42 ratio negatively correlated to cognitive tests SRT ($P = 0.002$) and SDMT ($P = 0.0001$).	Well-defined PD patients with long follow-up (DATATOP criteria) (98)
	Siderowf et al. (2010) (75)	45	11.0 (0.75)	↓ Aβ1-42 predicts significantly worse annual cognitive decline.	≥1-year follow-up (UKBBC) (89)

(continued)

Table 3 *(continued)*

Biomarker candidate	Author (year) (reference number)	Patients (*n*)	Disease duration, years	Results	Comments (Diagnostic criteria) (reference number)
	Compta et al. (2013) (76)	27	Median (IQR): 10 (7–15)	↓ baseline Aβ1-42 related to conversion to dementia and thinning of specific cortical regions on MRI.	(UKBBC) (89)
	Leverenz et al. (2011) (72)	22	Not specified	↓ Aβ1-42 related to poorer digit symbol performance. ↓ Aβ1-42/t-tau ratio correlated to poorer test performance on Digit Symbol and Category Fluency ($P = 0.02$).	(UKBBC) (90)
	Alves et al. (2014) (77)	104	Not specified	↓ baseline Aβ1-42 associated with marked risk of developing dementia ($P < 0.001$). Prediction of development of dementia in PD if [Aβ1-42] <443 pg/ml: ROC: sens 85% spec 60% AUC 0.725. Prediction of development of dementia in PD minor cognitively impaired if [Aβ1-42]<443 pg/ml: ROC: sens 100% spec 71% NPV 100%	5-year follow-up (UKBBC and Gelb's criteria) (89, 91)
Neurofilament Light Chain	Hall et al. (2012) (48)	90	Not specified	Positive correlation with H&Y score ($P = 0.002$)	(Gelb's criteria) (91)
UCHL-1	Mondello et al. (2014) (50)	PD 52/MSA 34/PSP 32/CBD 12	Median (IQR): 9 (5–17)	Positive correlation with H&Y score	(UKBBC) (89)
Apolipoprotein A1 and A2	Leverenz et al. (2011) (72)	22	Not specified	No association with cognitive abilities	(UKBBC) (90)
Apolipoprotein ε	Leverenz et al. (2011) (72)	22	Not specified	No association with cognitive abilities	(UKBBC) (90)
	Siderowf et al. (2010) (75)	45	11.0 (0.75)	≥1 apolipoprotein ε-allele associated with ↓ Aβ1-42.	≥1-year follow-up (UKBBC) (89)
F – Other					
Vitamin D-binding protein	Leverenz et al. (2011) (72)	22	Not specified	No association with cognitive abilities	(UKBBC) (90)

↑ = increase ↓ = decrease. Disease duration is recorded when collecting cerebrospinal fluid. It is defined as years since onset of Parkinson's disease symptoms and shown as mean (± standard deviation) unless otherwise specified. DA, dopamine; DHPG, dihydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; L-DOPA, L-3;4-dihydroxyphenylalanine; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HIAA, 5-hydroxyindoleacetic acid; NA, noradrenalin; PD, Parkinson's disease; DATATOP, Deprenyl and Tocopherol Antioxidative Therapy Of Parkinsonism; H&Y score, Hoehn & Yahr score; Q10, Coenzyme Q10; DAT-SPECT, dopamine transporter single-photon emission computed tomography; UKBBC, United Kingdom Brain Bank Criteria; UPDRS, Unified Parkinson's Disease Rating Scale; CSF, cerebrospinal fluid; flt3-ligand, Fms-related tyrosine kinase 3 ligand; IgG, immunoglobulin G; MRI, magnetic resonance imaging; PDD, Parkinson's disease dementia; sens, sensitivity; spec, specificity; BDNF, brain-derived neurotrophic factor; t-tau, total tau; Aβ, amyloid-β; ParkWest, Norwegian population-based study of the incidence; neurobiology; and prognosis of PD; PD-CIND, Parkinson's disease, cognitively impaired non-demented; MMSE, Mini-Mental State Examination; IQR, interquartile range; p-tau, phosphorylated tau; GMV, grey matter volume; SRT, selective reminding test; SDMT, symbol digit modalities test; ROC, receiver operating characteristics; AUC, area under the curve; NPV, negative predictive value; UCHL-1, ubiquitin carboxy-terminal hydrolase L1.

with measurement of ubiquitin and chromogranin I and II.

The fms-related tyrosine kinase 3 ligand (flt3-ligand) plays a role in the activation of the immune system and is increased in CSF of patients with amyotrophic lateral sclerosis (33). Unlike previous findings by Shi et al. (34) of increased flt3-ligand in MSA compared with PD, Silajdciz et al. (33) found no differences.

Disease severity and cognitive abilities – Regarding flt3-ligand, Silajdzic et al. (33) did find a positive correlation to the H&Y score in patients with PD.

Pisani et al. (35) found the CSF/serum IgG ratio to be increased in PD patients with H&Y score 2.4–4 and that the ratio correlated positively with the H&Y score, maybe indicating a dysfunctional blood–brain barrier in PD.

Growth factors

PD vs controls – Brain-derived neurotrophic factor (BDNF) plays a significant role in synaptic plasticity, and the presence of the minor allele (val66met) seems to be associated with a decrease in BDNF secretion and a significantly increased risk of developing L-DOPA-induced dyskinesia (36). Zhang et al. (30) found BDNF to be increased in AD and PD patients compared with controls and correct classification of PD, AD and controls was achieved optimally combining the eight biomarkers t-tau, BDNF, Il-8, amyloid-β1-42 (Aβ1-42), β2-microglobulin vitamin D-binding protein, apolipoprotein AII (apoAII) and apoE, correctly classifying 95% of 38 patients with PD and 95% of 38 controls compared with the clinical evaluation.

Sjögren et al. (37) found a protein associated with neuronal growth and regeneration called neuromodulin (or growth-associated protein 43; GAP-43) to be decreased in CSF of patients with PD.

PD vs other neurodegenerative diseases – Sjögren et al. (37) also found the aforementioned neuromodulin to be decreased in CSF of patients with PD compared with patients with Alzheimer's disease (AD).

Proteins involved in PD pathology

PD vs controls – The neuropathological criteria for PD include the identification of neuronal loss in the substantia nigra (pars compacta) and detection of Lewy pathology in the CNS neurons (38). The intraneuronal Lewy bodies (LB) contain aggregated and phosphorylated α-syn (39). The protein α-syn is mainly localized in presynaptic terminals and is presumed to play an important role in synaptic plasticity and the transport of vesicles to presynaptic terminals. Modification (e.g. phosphorylation), misfolding (e.g. oligomeric shapes) and accumulation of α-syn seem to play a key role in the pathogenesis of PD (40). Mutations in the α-syn gene are related to rare types of dominantly inherited PD (17, 41), further supporting the central role. Braak's hypothesis of spreading neuropathology from the brainstem to more central areas of the CNS gave rise to the idea of a prion-like spread of misfolded α-syn (40), for example from the intestinal nervous system via the vagal nerve to the CNS (42). Several studies using enzyme-linked immune assays (ELISA) have not shown differences between PD and control CSF (19, 43–47). Using other methods to quantify α-syn such as the more sensitive Luminex assay (17, 34, 48) and through time-resolved Förster's resonance energy transfer assay (TR-FRET) (49), but also ELISA (50–52), other studies have shown a decrease of t-α-syn in PD (see Table 1). Foulds et al. (47) did not find any increase in PD of phosphorylated α-syn (p-α-syn) or oligomeric α-syn (o-α-syn), but Tokuda et al. (45) found an increase in o-α-syn. Calculating the o-α-syn/t-α-syn ratio differed patients with PD from controls with a sensitivity of 89.3% and specificity of 90.6 with a 0.948 area under the curve (AUC).

Neurosin, a protease enzyme involved in the clearance of α-syn, was found by Wennström et al. (51) to be decreased in the CSF of patients with synucleinopathies, whereas Nielsen et al. (43) found no difference.

Another pathway of α-syn degradation is the ubiquitin-proteasome system. Mutations in the PARK2 gene coding for Parkin, an enzyme in the ubiquitin-proteasome system, are responsible for autosomal recessive juvenile parkinsonism (53). Mondello et al. (50) found another enzyme related to the ubiquitin-proteasome system, ubiquitin carboxy-terminal hydrolase (UCHL-1), to be decreased in PD CSF.

The breakdown of amyloid precursor protein (APP) creates the peptide amyloid-β1-42 (Aβ1-42) which can aggregate, creating neurotoxic amyloid plaques, that together with neurofibrillary tangles of tau-protein triggered by amyloid-β represents the pathological hallmark of Alzheimer's disease (AD) (54). In PD, α-syn might induce tau aggregation and interact with amyloid-β1-42, resulting in the aggregation of both peptides (55). Whereas several authors found no changes in

total tau (t-tau) or phosphorylated tau (p-tau) in PD (19, 43, 51, 56–58) (see Table 1), one study (59) found an increase in t-tau, another only in patients with PDD (60). Conversely, two studies (48, 61) found a decrease in p-tau and one found a decrease in t-tau (30).

Aβ species have also been examined (Table 1). Again the results point in different directions with some authors finding a decrease of Aβ1–15/16 in neurodegenerative diseases (62), decreased APPα, Aβ-38, 40 or 1-42 (43, 46, 51, 56, 58, 60) in PD, one only finding a decrease in cognitively impaired or demented PD patients (61). One study found an increase in Aβ1-42 (30), one study found no changes in APPα+β (63), whereas 5 other studies found no difference in Aβ1-42 concentrations (37, 48, 57, 59, 64).

Genetic mutations of transthyretin (TTR), a protein transporting thyroxin (T_4) and retinol (vitamin A), are related to familial amyloid polyneuropathy (FAP), and TTR seems to be related to diseases such as AD, PD and psychiatric disorders. One function seems to be prevention of the sequestering of Aβ species that are otherwise neurotoxic (65). Maarouf et al. (20) found a doubling of TTR in CSF of patients with PD, but the potential pathophysiological implications are not well understood.

In contrast to the findings of microgliosis in the substantia nigra of patients with PD, Olsson et al. (66) found the concentration of YKL-40, a marker of glial activation, to be significantly lower in patients with PD compared with controls. Although this might indicate decreased microglial activation, there were no neuropathological specimens available to support this observation.

Clusterin, also called apolipoprotein J, is a multifunctional protein among other things related to cell survival and protection from cytotoxic stress (59). Studying CSF from neuropathologically verified patients with PD, Maarouf et al. (20) found two times higher concentration of clusterin compared with normal controls; an increase was also found by Prikrylova Vranova et al. (59). Another apolipoprotein, ApoE, a carrier of cholesterol in the CNS whose allele ApoE ε4 is associated with a marked risk of dementia, has been shown to be either similar in PD and controls (58), increased (20) or decreased (30). The high-density lipoprotein (HDL) consists of ApoA1, which has both been found decreased (20) and unchanged (30) in patients with PD.

PD vs other neurodegenerative diseases – AD is a neurodegenerative disease with established CSF biomarkers, namely decreased Aβ1-42 and increased t-tau and p-tau. In accordance with the fact that amyloid plaques play a significant role in the pathology of AD, and probably to a higher extent than in PD, several studies have found patients with PD to have significantly higher Aβ1-42 concentrations than patients with AD (30, 37, 48, 58, 64, 67) (see Table 2). Furthermore, in PD compared with AD, a lower t-tau (30, 37, 48, 51, 56, 58) and p-tau (48, 51, 56, 58) have been shown in several studies. Neurosin was found by Wennström et al. (51) to be significantly lower in patients with PD compared with AD. Alves et al. (58) found lower concentrations of Apo E in PD compared with AD, a difference not found by Zhang et al. (30).

When comparing PD to APD, Abdo et al. (13) found significantly increased t-tau in PD compared with MSA-P. Conversely, Herbert et al. (19) and Süssmuth et al. (57) found a decrease in t-tau MSA patients and Süssmuth et al. also in PSP with predominant parkinsonism (PSP-P).

Using multivariate analysis incorporating the five biomarkers Aβ1-42, t-tau, p-tau, α-syn and neurofilament light chain (NfL, see later for description), Hall et al. (48) were able to distinguish PD from APD with a sensitivity of 85% and specificity of 92% (AUC 0.93).

Several studies find that t-α-syn cannot be used to distinguish PD from APD (19, 44, 45, 47, 48, 68) (see Table 2); however, Heegaard et al. (52) claimed that a low t-α-syn separates PD from DLB, MSA and PSP. Patients with PD also have lower concentrations of t-α-syn than patients with AD (48, 51, 68). Studying modified α-syn, Tokuda et al. (45) found higher amounts of oligomeric α-syn in PD compared with PSP and AD, whereas Foulds et al. (47) found patients with MSA to have higher concentrations of phosphorylated α-syn than PD and PDD patients.

The 542 amino acid protein NfL is the most abundant of the three subunits in the neurofilament structure. These proteins only exist in the CNS and thus function as a biomarker for axonal degeneration in acute nervous tissue damage and in neurodegenerative diseases (69). Both Abdo et al. (13), Bech et al. (67) and Hall et al. (48) found a significantly larger concentration of NfL in, respectively, patients with MSA, MSA, PSP and DLB patients, and MSA, PSP and CBD patients, maybe indicating a more rapid neurodegeneration in these diseases. Brettschneider et al. (70) found the concentration of the subunit neurofilament heavy chain (NfH) to be raised in patients with PSP (70) and Abdo et al. (13) in patients with MSA.

Olsson et al. (66) showed decreased levels of YKL-40 in PD compared with MSA, PSP and CBD patients, maybe indicating more glial activation in the latter diseases, although their concentrations did not differ significantly from controls.

Mondello et al. (50) showed that UCHL-1 concentrations were significantly lower in PD compared with MSA, PSP and CBD, also finding a correlation between α-syn and UCHL-1 concentrations, maybe indicating a connection in the pathogenic mechanism in PD.

Disease severity and cognitive abilities – Cognitive impairment is a common issue in patients with PD, and the risk of developing dementia in PD (PDD) is high, affecting especially executive and visuospatial functions (71), and increasing mortality.

An increasing amount of evidence points to the use of Aβ1-42 in the evaluation of cognitive abilities in PD (see Table 3). Alves et al. (58) found a significant positive correlation between Aβ concentration and memory performance, Nutu et al. (64) found a positive correlation with the MMSE score, and Leverenz et al. (72) found significant positive correlation between Aβ1-42 and digit symbol performance as well as a correlation between a decreased Aβ1-42/t-tau ratio and poorer performance on digit symbol and category fluency. Montine et al. (61) found Aβ1-42 to change dynamically, decreasing from PD to cognitively impaired PD (CI-PD) patients to PDD patients having the lowest concentrations. Compta et al. (60) showed that besides a positive correlation between Aβ1-42 and semantic and phonetic fluency, naming and visuospatial score, patients with PDD have significantly lower Aβ1-42 concentrations than non-demented patients. Combined with an MRI study, they also showed significant positive correlation in patients with PDD between Aβ1-42 concentrations and the grey matter volume of cerebellum and other cortical areas.

Zhang et al. (73) interestingly found the baseline Aβ1-42 to correlate negatively with baseline UPDRS score in patients from the DATATOP study.

Also tau pathology seems to be related to the cognitive decline in PD (see Table 3). Liu et al. (74) found t-tau and t-tau/Aβ1-42 ratio to be negatively correlated with the performance in the cognitive tests, selective reminding test (SRT) and symbol digit modalities test (SDMT). Compta et al. (60) found a negative correlation between t-tau, p-tau and memory, naming and visuospatial abilities. Parnetti et al. (56) found patients with PD to have significantly lower t-tau than patients with PDD, and again, it seems that there is a dynamic change from non-demented to demented; Montine et al. (61) found the percentage of patients with an abnormal t-tau/Aβ ratio increase from 15% in patients with PD, 29% in cognitively impaired PD patients to 45% of patients with PDD. Prikrylova Vranova et al. (59) also found patients with >2-year diagnosis of PD to have significantly higher t-tau and t-tau/Aβ1-42 ratio than PD <2 years.

Even though Mondello et al. (50) showed that synucleinopathies had the lowest concentration of UCH-L1, they also found an increase in UCH-L1 to be associated with an increased disease severity.

Prognostic value – Aβ1-42 can be used as a significant predictor for cognitive decline (see Table 3). Siderowf et al. (75) found that Aβ1-42 concentrations below a cut-off of 192 pg/ml were associated with a significantly larger annual decline in the Dementia Rating Scale (Version 2) (DRS-2) and furthermore found the mean DRS score after 2-year follow-up to be below the dementia cut-off in this group of patients. Compta et al. (76) also found patients with PD who converted to dementia at 18-month follow-up to have significantly lower Aβ1-42 concentrations at baseline, along with worse verbal learning, semantic fluency, visuoperceptual score and cortical thinning of specific brain areas in MRI. Alves et al. (77) found a low baseline Aβ1-42 to be related to the risk of developing dementia, but interestingly Liu et al. (74) on the same cohort (DATATOP trial patients, $n = 403$) did not find a correlation between Aβ1-42 and cognitive decline. Liu et al. did find a higher p-tau and p-tau/Aβ1-42 ratio to be related to a decline in SRT and visuospatial working memory and procedural speed.

Tau-protein has also been proposed as a marker for neural degeneration, and Zhang et al. (73) showed a negative correlation between baseline p-tau/t-tau ratio and the change rate in UPDRS and UPDRS motor score, and a positive correlation between the change rate of, respectively, t-tau and t-tau/Aβ1-42 ratio and the aforementioned modalities.

Others

PD vs controls – Using gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) metabolomics, Trupp et al. (46) was able to identify 4 principle drivers in the differentiation of PD and

controls, namely a decrease in creatinine, xylitol, 3-hydroxyisovaleric acid and tryptophan. A decrease in tryptophan might indicate an increased production of the excitotoxic 3-OH-kynurenine mentioned in section A (12).

Pisani et al. (35) found a higher CSF-/serum albumin ratio in patients with PD compared with controls, suggesting a compromised blood–brain barrier (BBB) in PD.

Disease severity and cognitive abilities – Pisani et al. (35) also found PD patients with H&Y score 2.5–4 to have an increased CSF-/serum albumin ratio compared with H&Y score 1–2, maybe indicating a progressive disruption of the BBB in PD. A compromised BBB might increase the amount of L-DOPA reaching the CNS, which may increase risk of L-DOPA-induced dyskinesia in later stages of the disease.

Discussion and conclusion

As this study shows, biomarker studies produce conflicting results. Sampling and analysis procedures differ from study to study, possibly obscuring significant differences between patients and controls. Methodological differences in the collection of CSF can cause significant changes in the concentrations of the biomarker in focus as underlined in two studies by Salvesen et al. (78) and Simonsen et al. (79). However, since 2009, international guidelines for the sampling and storage of CNS can be followed, thus allowing for reliable comparison between studies (80).

A single biomarker for Parkinson's disease?

In the search for biomarkers to distinguish PD from healthy controls, no single biomarker has been unequivocally significant. As a potential early biomarker, candidate for PD the metabolite DOPAC has been shown to be very sensitive in identifying PD with a specificity of 89%. Alpha-synuclein has a tendency to be decreased in the CSF of patients with PD, and an increased focus on modified versions such as oligomeric alpha-synuclein might be worthwhile pursuing, with the o-α-syn/t-α-syn ratio being one of the most sensitive and specific tests distinguishing PD from controls in this review, albeit only in one study with 28 patients with PD. As idiopathic Parkinson's disease is not a single homogenic disease but a clinical syndrome with different underlying disease mechanisms, the search for biomarkers has to reflect that difference. A broader potentially more fruitful approach is the use of untargeted metabolomics as performed by LeWitt et al. (12), which can serve both as a tool for more precise diagnosis and creating new hypotheses regarding the pathogenesis of PD.

Parkinson's disease and atypical Parkinson's disease

Neurofilament light chain functions as a biomarker for distinguishing PD from APD, with sufficient sensitivity and specificity to be of clinical value, and hopefully future studies will corroborate previous findings.

Cognitive impairment and disease severity

Amyloid and tau-protein analyses show a distinct pattern related to cognitive abilities, and they also have a prognostic value in the development of cognitive impairment and dementia that might also be of clinical value.

Although this review does not point towards a specific biomarker, many indicators of oxidative stress seem to be related to disease duration and severity, the latter being important for objectively measuring the effect of potential disease modifying drugs, and maybe future studies in this category can corroborate previous findings.

Future biomarkers for dyskinesia?

To our knowledge, only few CSF studies have focused on L-DOPA-induced dyskinesia (LID), which is a common and oftentimes debilitating side effect to prolonged L-DOPA treatment. Identifying PD patients with a risk of developing LID might make it possible to adjust the medical treatment in earlier disease stages, preventing or postponing the debut of LID. Lunardi et al. (81) found an increased HVA/dopamine ratio in dyskinetic patients, which indicates an increased dopamine metabolism in non-dopaminergic cells, as catechol-O-methyltransferase (COMT), the enzyme responsible for the production of HVA, is not present in dopaminergic neurons. Apart from markers of dopamine turnover, other biomarkers may be identified by metabolomics and assays for BDNF and vascular endothelial growth factor (VEGF), associated with abnormal plasticity in animal models of LID (82).

In future, standardized longitudinal studies with a wider biomarker focus (e.g. metabolomics), especially including patients with possible premotor symptoms of PD, are expected to play an important role in the ongoing search for PD biomarkers for early diagnosis and prognosis, and will help in elucidating the

pathological mechanism of the Parkinson's disease complex.

Acknowledgments

No acknowledgements.

Conflict of interest

The authors declare there are no conflict of interests.

Sources of funding

No sources of funding.

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APPENDIX II

Paper 2

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2017

doi: 10.1111/jnc.13997

ORIGINAL ARTICLE

Cerebrospinal fluid levels of catecholamines and its metabolites in Parkinson's disease: effect of L-DOPA treatment and changes in levodopa-induced dyskinesia

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Abstract

Levodopa (L-DOPA, L-3,4-dihydroxyphenylalanine) is the most effective drug in the symptomatic treatment of Parkinson's disease (PD), but chronic use initiates a maladaptive process leading to L-DOPA-induced dyskinesia (LID). Risk factors for early onset LID include younger age, more severe disease at baseline and higher daily L-DOPA dose, but biomarkers to predict the risk of motor complications are not yet available. Here, we investigated whether CSF levels of catecholamines and its metabolites are altered in PD patients with LID [PD-LID, $n = 8$)] as compared to non-dyskinetic PD patients receiving L-DOPA (PD-L, $n = 6$), or not receiving L-DOPA (PD-N, $n = 7$) as well as non-PD controls ($n = 16$). PD patients were clinically assessed using the Unified Parkinson's Disease Rating Scale and Unified Dyskinesia Rating Scale and CSF was collected after overnight fasting and 1–2 h after oral intake of L-DOPA or other anti-Parkinson medication. CSF catecholamines and its metabolites were analyzed by HPLC with electrochemical detection. We observed (i) decreased levels of dihydroxyphenylacetic acid (DOPAC) and homovanillic acid in PD patients not receiving L-DOPA (ii) higher dopamine (DA) levels in PD-LID as compared to controls (iii)

Received December 29, 2016; revised manuscript received February 9, 2017; accepted February 15, 2017.

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Abbreviations used: 3-OMD, 3-*O*-methyldopa; 5-HIAA, 5-hydroxyindoleacetic acid; 6FD, 6-^{18}F-fluoro-L-DOPA; AADC, aromatic amino acid decarboxylase; AD, Alzheimer's disease; AUC, area under the curve; BDNF, brain-derived neurotrophic factor; DA, dopamine; DAergic, dopaminergic; DAT, dopamine transporter; DAT-ir, DAT-immunoreactivity; DLB, Lewy body dementia; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; LC, locus coeruleus; LED, levodopa equivalent dose; LID, levodopa-induced dyskinesia; MAO, monoamine oxidase; MDS-UPDRS, Movement Disorder Society Unified Parkinson's Disease Rating Scale; MHPG, 3-methoxy-4-hydroxyphenylglycol; MMSE, Mini-Mental State Examination; MOCA, Montreal Cognitive Assessment; NAergic, noradrenergic; PCA, perchloric acid; PD-LID, Parkinson's disease patients receiving L-DOPA, dyskinetic; PD-L, Parkinson's disease patients receiving L-DOPA, non-dyskinetic; PD-N, Parkinson's disease patients not receiving L-DOPA; PD, Parkinson's disease; PET, positron emission tomography; ROC, receiver operating characteristics; UDysRS, unified dyskinesia rating scale.

higher DA/L-DOPA and lower DOPAC/DA ratio's in PD-LID as compared to PD-L and (iv) an age-dependent increase of DA and decrease of DOPAC/DA ratio in controls. These results suggest increased DA release from non-DA cells and deficient DA re-uptake in PD-LID. Monitoring DA and DOPAC in CSF of L-DOPA-treated PD patients may help identify patients at risk of developing LID.

Keywords: aging, biomarkers, dopamine, HPLC, metabolites, neurodegeneration.
J. Neurochem. (2017) 10.1111/jnc.13997

Parkinson's disease (PD) is a common age-related neurodegenerative disease with a prevalence of about 1% in people over 60 years and up to 4% in the oldest age-groups (de Lau and Breteler 2006). Diagnosis is based on clinical symptoms including bradykinesia, and either rigidity, resting tremor, or both (Postuma *et al.* 2015). The dopamine precursor L-DOPA is the most effective drug in the symptomatic treatment of PD, but chronic use is associated with serious motor complications, e.g. fluctuations (wearing off, on-off) and dyskinesia (chorea, dystonia and athetosis). This phenomenon is called L-DOPA-induced dyskinesia (LID), and is seen in the majority of patients (80% within 5 years) (Bastide *et al.* 2015). Attempts to alleviate established LID by reducing L-DOPA dosage often result in impaired control of Parkinsonian symptoms. Balancing this trade-off is a major challenge in advanced PD (Marsden and Donaldson 2012). Risk factors for earlier onset of LID include younger age, greater disease severity at baseline, higher daily L-DOPA dosage and a polymorphism in brain derived neurotrophic factor (Rascol *et al.* 2006; Olanow *et al.* 2013; Cheshire *et al.* 2014). If PD patients with increased risk for LID could be identified, their anti-Parkinsonian medication could be adjusted in an early phase, which may prevent or postpone the onset of LID. Thus, there is a clinical need to identify biomarkers for LID.

In drug-naïve PD or following drug washout, CSF levels of the dopamine (DA) metabolite 3,4-dihydroxyphenylacetate (DOPAC) are significantly decreased as compared to age-matched controls (Goldstein *et al.* 2008, 2012), which is indicative of the reduced synthesis and metabolism of DA in brains of PD patients not currently receiving L-DOPA. The DA metabolite homovanillic acid (HVA) may also be reduced in PD CSF, but in contrast to DOPAC, it is not a reliable marker for central DA deficiency (Wood 1980; LeWitt *et al.* 1992, 2011; Chia *et al.* 1993; Meiser *et al.* 2013).

Most treatment strategies in PD are aimed at enhancing dopaminergic (DAergic) transmission in the basal ganglia. Treatment with the DA precursor L-DOPA *increases* CSF levels of catecholamines and its metabolites (Raftopoulos *et al.* 1996), whereas treatment with a monoamine oxidase B (MAO-B) inhibitor reduces CSF levels of the acidic metabolites DOPAC and HVA (Heinonen *et al.* 1993) Parkinson study group 1995), but increases interstitial DA levels – due to the combined effects of blocked catabolism and inhibition of DA-reuptake by an active metabolite of the MAO-B-inhibitor (Knoll 1978). Chronic treatment with the D2/D3-recepter agonist pramipexole increases DOPAC and HVA levels and DA turnover in mice (Schulte-Herbruggen *et al.* 2012), possibly due to downregulation of presynaptic D2-receptors.

There is some evidence that chronic L-DOPA treatment increases angiogenesis and thereby access of L-DOPA to the brain (Ohlin *et al.* 2011; Janelidze *et al.* 2015). Apart from this, L-DOPA-induced DA release in the striatum may be handled differently in LID versus non-dyskinetic PD patients, giving rise to excessive stimulation of postsynaptic DA receptors. Such changes have been observed in experimental models of LID (Bastide *et al.* 2015), but only a few clinical studies have addressed possible presynaptic differences in LID versus non-dyskinetic PD. Two of these studies reported increased DA turnover in dyskinetic patients, either based on neurochemical analysis of CSF (Lunardi *et al.* 2009) or 6-^{18}F-fluoro-L-DOPA positron emission tomography (PET) scans (Sossi *et al.* 2006). Studies using 6-^{18}F-fluoro-L-DOPA-PET have further suggested that downregulation of the dopamine transporter (DAT) beyond the degree expected based on disease severity, is an independent predictor of the development of LID (Sossi *et al.* 2007).

While previous CSF studies on catecholamines in PD collected CSF from drug-naïve patients or after drug washout, here we collected CSF from PD patients at 1.5–2 h after oral intake of L-DOPA [+ peripheral aromatic L-amino acid decarboxylase inhibitor (AADC-I)] intake; i.e. the time interval where peak plasma levels are reached (Okereke *et al.* 2004; Huot *et al.* 2012). Using this approach, we studied the L-DOPA-induced effects on DA metabolism in non-dyskinetic and dyskinetic PD patients. After collecting CSF, patients were clinically assessed, using the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part III and the Unified Dyskinesia Rating Scale (UDysRS) (Goetz *et al.* 2008a,b).

The aim of this study was to investigate whether L-DOPA, NA, DA and its metabolites are altered in LID compared to non-dyskinetic PD patients and controls.

Materials and methods

Patient and control recruitment

PD patients and controls were recruited from three different neurological clinics in the Region of Southern Denmark (Hospitals

in Sønderborg, Odense) as well as a private neurological practice (Esbjerg) one neurological clinical in Region Zealand (Roskilde). The study included 20 PD patients and one Lewy body dementia (DLB) patient, all diagnosed by movement disorder specialists using the UK Brain Bank Criteria for idiopathic PD (Hughes *et al.* 1992). Since DLB, like PD, is a synucleinopathy, we included this patient in the PD group. Controls were patients referred by their physician or practicing neurologist for further neurological examination, and lumbar puncture was part of the pre-determined diagnostic set-up. The following referring diagnoses were noted: headache $n = 3$, dementia $n = 3$, multiple sclerosis $n = 3$, vertigo $n = 1$, ataxia $n = 1$, trigeminal neuralgia $n = 1$, unspecified sleep disorder $n = 1$, neuroborreliosis $n = 2$. Two controls were diagnosed as having Alzheimer's disease. One had a possible brain stem or cerebellar disorder, one post concussion syndrome, and one had a potential sleep disorder, but was discharged without a specific diagnosis. The rest of the controls were not diagnosed with a neurological disorder. All PD patients and controls were informed in writing and orally about the project, and gave written consent prior to participation in the project, which had been approved by the local ethics committee in the Region of Southern Denmark (S-20130098). The study conforms to The Code of Ethics of the World Medical Association.

CSF sampling

Lumbar punctures (LP) on PD patients were performed between 09.30 and 10.00 am after overnight fasting (19 of 21 patients). Patients using L-DOPA were instructed to (when possible) take their morning dose at 08.00 am, but time of intake varied from 05.30 am to 08.00 am. For PD patients the procedure was performed in a sitting position, using an atraumatic Pencan® (B. Braun Medical Inc., Frederiksberg, Denmark) (0.53 × 88 mm) needle at the L3/L4 level. CSF was withdrawn as follows:

Step 1: 2–3 mL CSF for routine analysis.
Step 2: 2 × 360 μL CSF was pipetted into two brown Saarstedt tubes containing 40 μL of 1 M perchloric acid (PCA) with antoxidants (0.2 g/L Na_2SO_2, 0.05 g/L Na_2EDTA), immediately placed on dry ice and stored at −80°C.

For controls, the procedure was performed between 09.30 am −12.15 pm, using an atraumatic needle. A subgroup of controls had not been fasting before withdrawal of CSF. Six out of 16 control patients had the procedure done in a sitting position without measurement of the CSF pressure, 10 had the procedure done lying on the side with CSF pressure measurement before withdrawing CSF as part of their investigation. Apart from this the same steps for sampling CSF were followed as described above.

Clinical rating

Clinical rating was performed on the same day as the LP procedure. PD motor symptom severity was rated in the ON-stage using the MDS-UPDRS part III (Goetz *et al.* 2008b). For patients using L-DOPA it was performed approximately 50 min after intake. The procedure was recorded on video for later assessment (apart from evaluating rigidity). UDysRS (Goetz *et al.* 2008a) objective score procedure was performed with video recording for later rating. For patients using L-DOPA, it was performed approximately 50 min after drug intake. Cognitive rating was done using the Mini-Mental State Examination and Montreal Cognitive Assessment scales.

Subgroups of PD patients

PD patients were divided into three groups: patients not receiving L-DOPA (PD-N, $n = 7$), patients receiving L-DOPA but without dyskinesia (PD-L, $n = 6$), and patients receiving L-DOPA and having dyskinesia (PD-LID, $n = 8$). PD-LID patients were assigned to this group either with a positive UDysRS objective score ($n = 6$), clearly dyskinetic presentation but recruited before the use of the UDysRS ($n = 1$), or amnestic description of LID symptoms ($n = 1$). Unless otherwise stated, PD-N, PD-L and PD-LID will be used to describe the separate groups. A flowchart of the clinical work process is shown in fig.1.

HPLC analysis of catecholamines and its metabolites

On the first day of HPLC analysis, CSF-containing PCA was thawed on ice and centrifuged at 20,627 *g* for 20 min at 4°C. The supernatant was used for HPLC analysis. A small volume (50 μL) of unpurified supernatant was used for analysis of 3-methyl-dopa (3-OMD), 3-methoxy-4-hydroxyphenylglycol (MHPG), DOPAC, HVA, (and 5-HIAA). 250–700 μL supernatant was used for extraction of catecholamines (purification) using RECIPE ClinRep. Plasma Assay Kit (RECIPE, Munich, Germany). Before cleaning up, the pH of the PCA-containing supernatant was adjusted to pH 5–6 using 5–8 μL 10 M NaOH.

L-DOPA, noradrenaline, dopamine, serotonin, and its respective metabolites 3-OMD, MHPG, DOPAC, HVA, and 5-HIAA were analyzed by HPLC with electrochemical detection, essentially as described (Gramsbergen *et al.* 2002; Lambertsen *et al.* 2012), but with some modifications allowing detection and quantification of all compounds of interest.

The Merck-Hitachi HPLC system consisted of an L-7100 pump, an L-7200 autosampler, a D-7000 interface and an electrochemical detector with in-built column oven (Decade; Antec, Leiden, The Netherlands), connected to a computer equipped with D-7000 version 4.0 chromatography software (Hitachi, San José, California, USA). The electrochemical cell was set to 750 mV versus Ag/AgCl. The mobile phase was pumped at a flow rate of 0.9 mL/min through a Waters Spherisorb S5 ODS2 guard column (4.6 × 30 mm; Waters Corp, Milford, MA, USA) and a Waters Spherisorb S3 ODS2 cartridge analytical column (4.6 × 150 mm; Waters Corp). A mixture of external standards of monoamines and metabolites (Sigma-Aldrich Denmark A/S, Brøndby, Denmark) was injected (0.25–0.5 pmol per standard) to identify and quantify the compounds of interest in the CSF samples.

For analysis of L-DOPA, 3-OMD, MHPG, DOPAC, HVA, and 5-HIAA in unpurified PCA extracts, samples were diluted 10× in mobile phase and 2, 5 or 20 μL was injected into the HPLC system, using a mobile phase containing 2% methanol, 0.1 M sodium acetate, 0.035 mM disodium ethylenediaminetetraacetate (Na_2EDTA), octanesulfonic acid (0.176 mM), pH 3.15 adjusted with glacial acetic acid.

For analysis of noradrenaline and dopamine, 50 μL of undiluted purified samples (RECIPE, Munich, Germany) were injected into the HPLC system using a mobile phase containing 3% acetonitrile, 94 mM NaH2PO4, 0.71 mM octanesulfonic acid, 0.2 mM Na2EDTA, pH 3.2 adjusted with 1 M phosphoric acid. Recovery

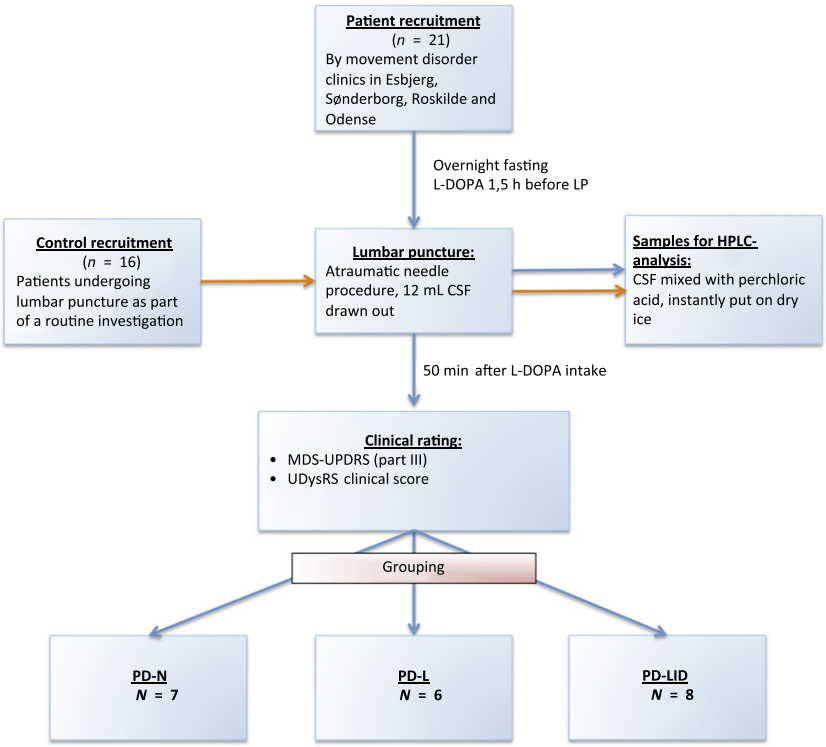

Fig. 1 Flowchart showing the clinical work process. LP; lumbar puncture, MDS-UPDRS; Movement Disorder Society modified Unified Parkinson's Disease Rating Scale, UDysRS; Unified Dyskinesia Rating Scale, PD-N; Parkinson's disease patients not treated with L-DOPA, PD-L; Non-dyskinetic patients treated with L-DOPA, PD-LID; Dyskinetic patients treated with L-DOPA.

of internal standard, 3,4-dihydroxybenzoic acid (added to the RECIPE cleaning up columns), was assessed by injecting 5 μL of purified sample and revealed a recovery of 72 ± 2%. Obtained catecholamine data were not adjusted for recovery of the cleaning up procedure.

Control and PD samples were not blinded during processing for catecholamines. Even though we performed no blinding in regards to samples being from patients or controls, L-DOPA treatment and clinical scores (UDysRS, UPDRS III) of PD patients were blinded during the neurochemical processing (Fig. 1).

Statistical analysis

Power analysis was not performed, but originally we aimed to collect plasma and CSF samples of about 25 dyskinetic and 25 non-dyskinetic PD patients and 25 age-matched controls. However, due to difficulties in obtaining CSF samples from PD patients, we decided to proceed analysing the rather small sample numbers obtained within the time-limited recruitment phase of the project. Statistical calculations were performed, using Stata/IC 14.2 (StataCorp, College Station, Texas, USA) for Mac. Comparisons between two groups were done using *t*-test and Mann–Whitney *U*-test for parametric and non-parametric numerical variables, respectively, and two-sample Kolmogorov–Smirnov for binary variables.

Comparisons between more than two groups were done using ANOVA for normally distributed data and Kruskal–Wallis equality-of-populations rank test for non-parametric numerical values followed by Dunn's Pairwise Comparison (without adjustment) for multiple comparison. For binary values Fisher's exact test was used. A *p*-value of 0.05 was used as the level of statistical significance. Figures were made using Prism 7.0 for Mac OS X (GraphPad Software, Inc., La Jolla, California, USA).

All statistics were performed with and without inclusion of the DLB patient in the PD-L group and with and without inclusion of two Alzheimer's disease patients in the control group.

Results

Demographic analysis

Patient data are summarized in Table 1. The average age of the control group was about 10 years younger than the average age of PD patients in PD-N and PD-L, but within the same age-range as the PD-LID patients (Table 1). There was a male preponderance in the combined PD group, whereas the gender distribution reflected the general population in the control group. CSF-protein levels were significantly lower in controls, but these levels are not correlated with CSF catecholamine and metabolite levels in either group. PD-L patients had significantly higher MDS-UPDRS part III scores than PD-N. The latter group was not treated with L-DOPA, accordingly the calculated, lower daily L-DOPA-equivalent dose in this group is based on the use of dopamine D2 receptor agonists (all seven in PD-N group, ropinirol $n = 5$, pramipexole $n = 2$) and in all but two patients combined with MAO-B-inhibitor treatment (Selegeline). PD-LID patients were treated with the highest levodopa equivalent dose and had a significantly lower age of debut of disease symptoms compared to PD-N and PD-L patients.

The Montreal Cognitive Assessment score testing executive function is slightly decreased in all PD patients with no significant difference between the subgroups. No difference

Table 1 Clinical characteristics of the patient and control groups

	Control	PD-N	PD-L	PD-LID	PD total
Number of cases (*n*)	16	7	6	8	21
Age (years)	55.1 ± ll[a]	64.3 ± 5.1	67 ± 13.3[b]	58.5 ± 8.3	62.9 ± 9.5
Sex (F/M)	8/8	3/4	0/6	2/6	5/16
CSF protein (g/L)	0.34 ± 0.08[c]	0.5 ± 0.18	0.46 ± 0.16	0.45 ± 0.15	0.47 ± 0.16
UPDRS III		19 ± 7.3[d]	37.8 ± 8.4	29.5 ± 13.3	28.4 ± 12.4
UDysRS objective score		0	0	7.9 ± 5	3.1 ± 4.9
Symptom duration (years)		3.4 ± 2[e]	6.4 ± 4	9 ± 4.7	6.4 ± 4.3
LED (mg)	10 ± 40[f]	320.7 ± 106.8[g]	525 ± 150[h]	848.4 ± 292.3	580.1 ± 303.7
MOCA		27.7 ± 2.1	25.8 ± 2.5	25.9 ± 1.6	26.6 ± 2.1
MMSE		29.1 ± 1.5	27.3 ± 4.2	29.3 ± 0.7	28.7 ± 2.4

PD-N, Parkinson's disease not receiving L-DOPA; PD-L, Non-dyskinetic Parkinson's disease receiving L-DOPA; PD-LID, Dyskinetic Parkinson's disease receiving L-DOPA; L-DOPA, Levodopa; F, female; M, male; UPDRS, Unified Parkinson's disease rating scale; UDysRS, Unified dyskinesia rating scale; LED, Daily levodopa equivalent dosage; MOCA, Montreal Cognitive Assessment; MMSE, Mini mental state examination; DLB, Lewy body dementia; AD, Alzheimer's disease. Numerical values are described as mean ± SD.

[a] $p = 0.0264$ versus PD total, $p = 0.0096$ versus PD-L, $p = 0.0211$ versus PD-N when excluding AD and DLB cases.

[b] $p = 0.0454$ versus PD-LID only when including DLB case.

[c] $p = 0.0056$ versus PD total, $p = 0.0048$ versus PD-N, $p = 0.0424$ versus PD-L, $p = 0.0438$ versus PD-LID.

[d] $p = 0.013$ versus PD-L.

[e] $p = 0.0035$ versus PD-LID, $p = 0.018$ versus PD-L when excluding DLB case.

[f] One control received Ropinirol for restless legs.

[g] $p = 0.0419$ versus PD-L, $p = 0.0001$ versus PD-LID.

[h] $p = 0.0397$ versus PD-LID only when including DLB case.

is found in the Mini-Mental State Examination score across the PD groups.

CSF catecholamines and metabolites

In Fig. 2 the measured CSF levels of L-DOPA, DA, NA and the metabolites 3-OMD, DOPAC, HVA, MHPG and 5-HIAA are depicted. L-DOPA treatment significantly increased L-DOPA, 3-OMD and DA levels in the PD-L and PD-LID groups as compared to controls. DOPAC and HVA levels were also elevated by L-DOPA treatment, reaching significance in comparison with PD-N. In the latter PD group, we observed significantly decreased L-DOPA, DA, DOPAC and HVA levels as compared to controls.

In PD-L CSF levels of NA were significantly increased as compared to controls and PD-N. In PD-LID, NA and MHPG levels were not different from controls. In PD-N we observed lower MHPG levels as compared to PD-LID. The levels of 5-HIAA are not different between controls or any of the PD groups.

In Fig. 3 receiver operating characteristics between PD-N and controls are shown. DA, DOPAC and HVA levels significantly separate the two groups.

Ratios

In Fig. 4 ratios of DA/L-DOPA, DOPAC/DA, HVA/DA and MHPG/NA in controls and the three PD groups are depicted. In comparison to controls, we observed that the DA/L-DOPA-ratio is reduced in PD-L but not in PD-LID. The DOPAC/DA and HVA/DA-ratio's are reduced in both PD-L and PD-LID as compared to controls and PD-N. The MHPG/NA-ratio is only decreased in PD-L, but not in PD-LID We observed that DA/L-DOPA (increased), DOPAC/DA (reduced) and MHPG/NA (increased) are altered in PD-LID as compared to PD-L.

Age-dependent effects in controls

In Fig. 5, correlations between age and DA, the DOPAC/DA-ratio and 5-HIAA are shown. We found a significant age-dependent increase in DA, and significant age-dependent decreases in DOPAC/DA-ratios and 5-HIAA levels.

Discussion

The main findings of this neurochemical study of CSF of PD patients and controls are:

(i) Significant differences in L-DOPA-induced changes of dopamine and its metabolites in PD-LID versus PD-L;
(ii) Reduced DOPAC and HVA levels in PD not treated with L-DOPA;
(iii) An age-related increase of dopamine and reduced DOPAC/DA ratio in controls

These findings are discussed below.

L-DOPA-induced changes in dyskinetic and non-dyskinetic PD patients

L-DOPA treatment has a profound effect on CSF levels of L-DOPA and its metabolites 3-OMD and dopamine. It is clear

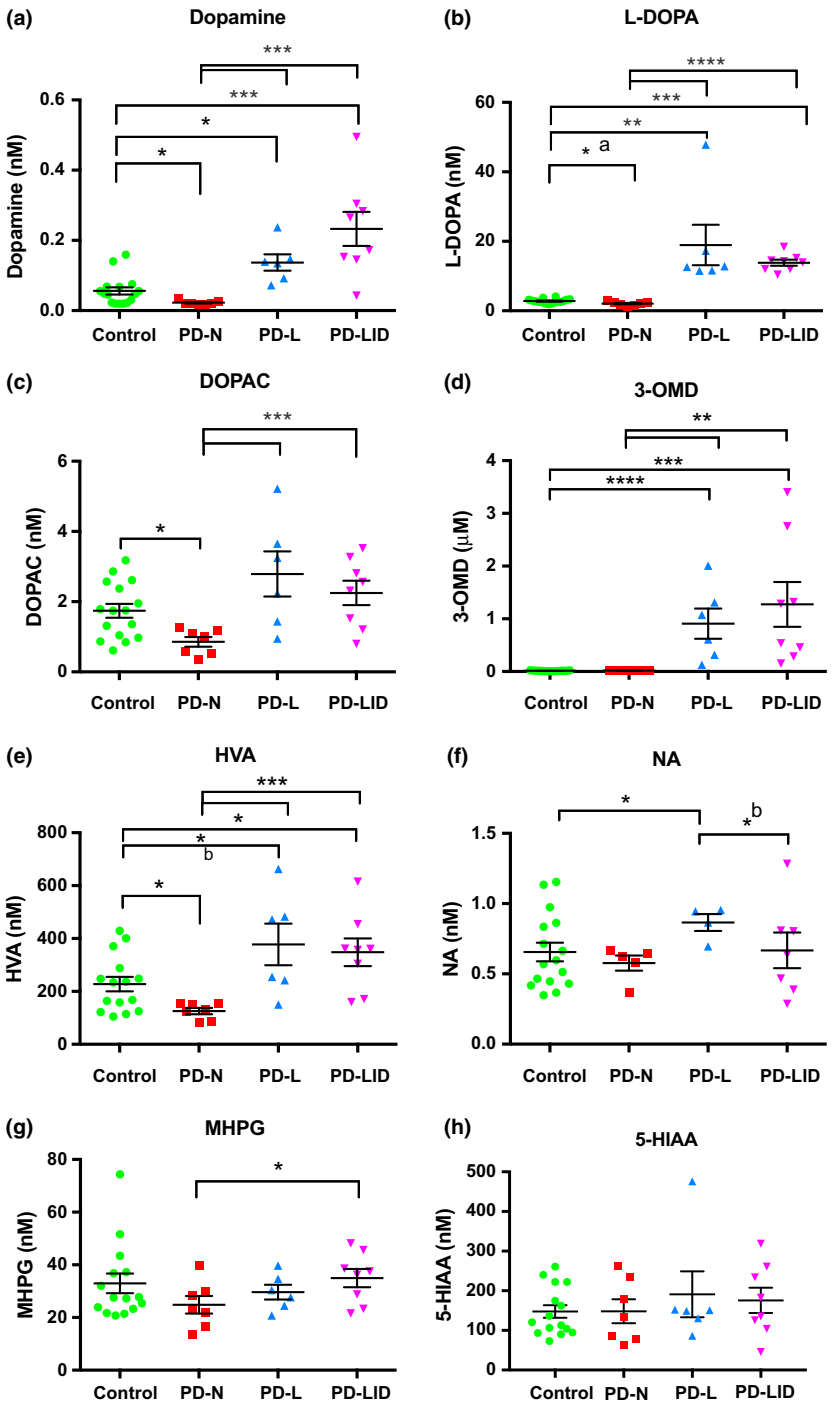

Fig. 2 CSF levels of dopamine, L-DOPA, dihydroxyphenylacetic acid (DOPAC), 3-*O*-methyldopa (3-OMD), homovanillic acid (HVA), noradrenaline (NA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5-hydroxyindoleacetic acid (5-HIAA) in controls and PD. PD-N, Parkinson's disease non-L-DOPA-treated; PD-L, Parkinson's disease, L-DOPA-treated, non-dyskinetic; PD-LID, Parkinson's disease, L-DOPA-treated, dyskinetic; DLB, Lewy body dementia; AD, Alzheimer's disease. Brackets represent mean and SEM. (a) ANOVA $p = 0.0001$, (b) ANOVA $p = 0.0001$, (c) ANOVA $p = 0.0107$, (d) ANOVA $p = 0.0001$, (e) ANOVA $p = 0.002$, (f) ANOVA not significant, (g) ANOVA not significant, and (h) ANOVA not significant. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$ $^{****}p < 0.0001$. a: Only significant when excluding AD patients. b: Only significant when including DLB patient.

from our CSF analysis that L-DOPA treatment compensates for the loss of L-DOPA and DA as observed in the PD-N group. Our findings of increased L-DOPA, 3-OMD, DA, DOPAC, and HVA in L-DOPA-treated PD patients are in line with previous CSF studies (Chia *et al.* 1993; Raftopoulos *et al.* 1996), but in the present study we looked also for differences between the dyskinetic (PD-LID) and non-dyskinetic, L-DOPA-treated patients (PD-L).

Although PD-LID received higher daily doses of L-DOPA (Table 1) we did not detect higher L-DOPA CSF levels in this dyskinetic group. 3-OMD has a longer half life than L-DOPA, which accumulates during L-DOPA treatment (Bartholini *et al.* 1972). Accordingly, CSF 3-OMD levels are dramatically increased in L-DOPA-treated patients (low μM range), with a tendency toward higher 3-OMD levels in dyskinetic patients. In L-DOPA- treated patients we measured

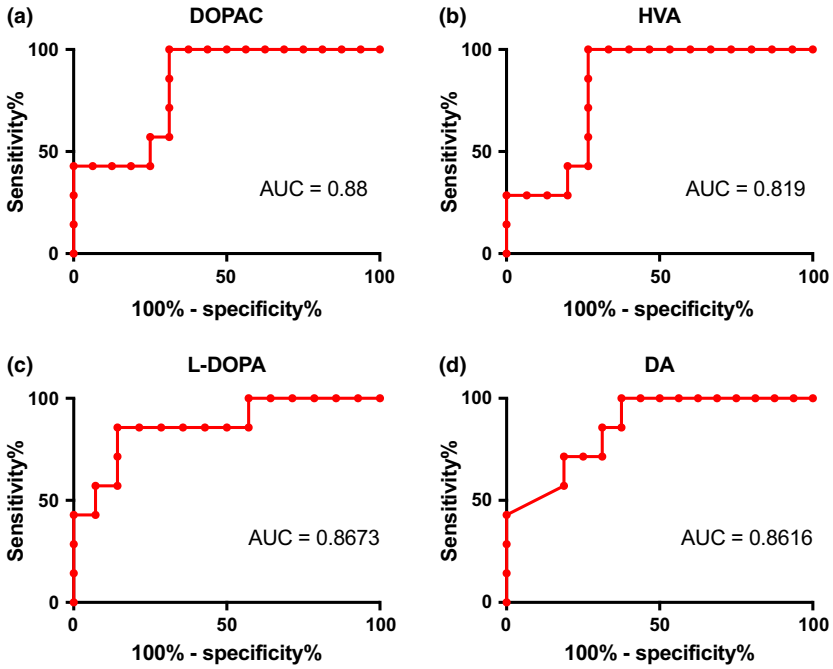

Fig. 3 Receiver operating characteristics analysis between controls and Parkinson's disease patients not receiving L-DOPA (PD-N). (a) $p = 0.0134$ (Alzheimer's disease cases excluded), (b) $p = 0.0182$, (c) $p = 0.0072$, and (d) $p = 0.0068$.

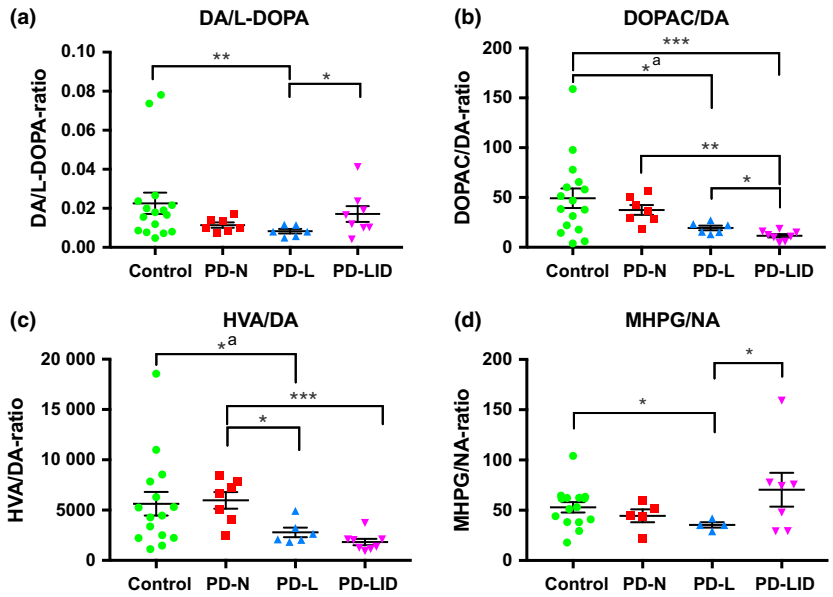

Fig. 4 CSF dopamine (DA)/L-DOPA-, dihydroxyphenylacetic acid (DOPAC)/DA-, homovanillic acid (HVA)/DA-, and 3-methoxy-4-hydroxyphenylglycol (MHPG)/NA-ratios in controls and patient groups. PD-N, Parkinson's disease non-L-DOPA-treated; PD-L, Parkinson's disease, L-DOPA-treated, non-dyskinetic; PD-LID, Parkinson's disease, L-DOPA-treated, dyskinetic; DLB, Lewy body dementia; AD, Alzheimer's disease. Brackets represent mean and SEM. (a) ANOVA not significant, (b) ANOVA $p = 0.0018$, (c) ANOVA $p = 0.0019$, and (d) ANOVA not significant. $*p < 0.05$ $**p < 0.01$ $***p < 0.001$. a: Only significant when excluding AD patients and DLB patient.

L -DOPA levels around 20 nM, which is almost a factor 50 less than 3-OMD. However, L-DOPA levels were measured after cleaning up with the RECIPE kit, which has a low recovery for L-DOPA, whereas 3-OMD was measured directly in PCA extracts (without further cleaning up). Slightly higher 3-OMD levels in dyskinetic patients are in line with the generally higher L-DOPA dose these patients receive (see Table 1). Higher plasma levels of 3-OMD in dyskinetic versus non-dyskinetic L-DOPA-treated patients has been reported previously (Feuerstein *et al.* 1977).

L-DOPA-induced dyskinesia (LID) is the result of maladaptive presynaptic and postsynaptic changes due to chronic L-DOPA treatment in advanced stages of PD (Bastide *et al.* 2015). CSF levels of catecholamines and their metabolites in samples taken at peak plasma levels of L-DOPA, may be used as markers of the integrity of the presynaptic DA-ergic system, including reuptake of dopamine.

In this study we observed about twofold elevated DA levels in L-DOPA-treated, non-dyskinetic patients and about fourfold increases in PD-LID, which for the latter are significantly different from controls. It is likely that such

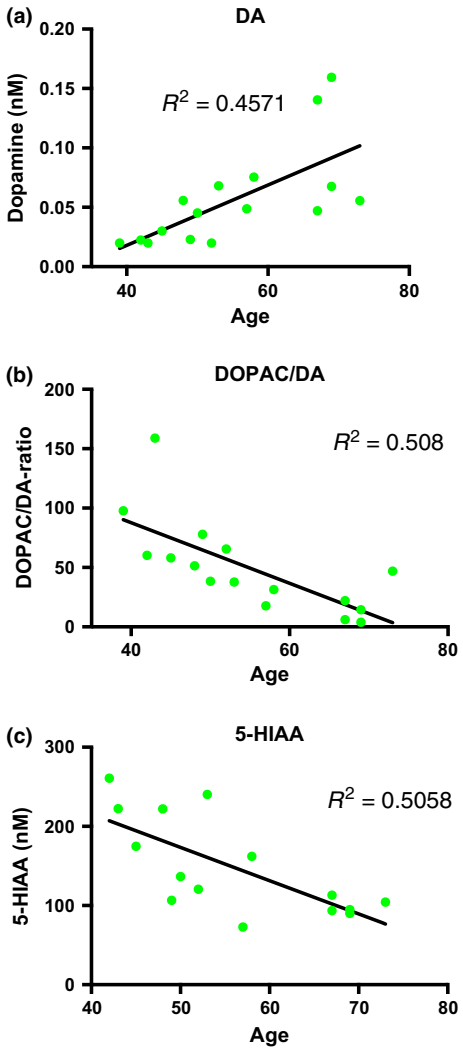

Fig. 5 Correlation between age and CSF dopamine (DA) (a), dihydroxyphenylacetic acid (DOPAC)/DA-ratio (b) and 5-HIAA levels (c) in controls. (a) $p = 0.004$, (b) $p = 0.001$, and (c) $p = 0.000$.

elevated DA levels also occur at the postsynaptic DA receptor level in the striatum, which may cause excessive stimulation of sensitized D1 receptors, resulting in the adverse dyskinetic effects (Cenci and Konradi 2010; Murer and Moratalla 2011). Underlying causes of the elevated CSF DA level in dyskinetic patients may include predominant release of DA from non-DAergic cells, for instance 5-HT terminals (Carta and Bezard 2011; Bezard *et al.* 2013). Higher levels of DA might also be due to a down regulation of dopamine transporters, possibly in excess of the degree expected based on the loss of DAergic terminals alone (Sossi *et al.* 2007, 2009).

It has also been proposed that chronic L-DOPA treatment stimulates angiogenesis and makes the blood brain barrier more permeable for L-DOPA, causing higher elevations of L-DOPA and DA in the CNS (Ohlin *et al.* 2011; Janelidze *et al.* 2015). However, we did not observe higher L-DOPA levels in LID patients. Furthermore, we observed a significantly higher DA/L-DOPA ratio in PD-LID versus PD-L, suggesting higher decarboxylation rate of L-DOPA in dyskinetic patients, probably due to AADC activity in 5-HT neurons (Carta *et al.* 2007).

Our study showed that L-DOPA treatment has a marked effect on HVA and DOPAC levels, which are almost doubled compared to controls and about 4 times higher than PD patients not treated with L-DOPA. DOPAC is produced intracellularly in the terminals of DAergic neurons. If dyskinetic patients have a more severe loss of DAergic nigrostriatal terminals, this will result in lower DOPAC levels (De Deurwaerdere *et al.* 2016) and in combination with the increased DA in PD-LID, we found a significantly lower DOPAC/DA ratio in PD-LID versus PD-L. Thus, the present results may indicate increased DA production in non-DAergic cells in combination with severely compromised re-uptake.

In PD, noradrenergic cell loss in the locus coeruleus (LC) precedes and may be equal or even greater than nigrostriatal dopaminergic cell loss (Zarow *et al.* 2003). In the present study we observed an increased NA turnover (MPHG/NA-ratio) in PD-LID compared to PD-L. This difference in NA turnover is in agreement with increased firing rates of LC neurons observed in the 6-hydroxydopamine lesioned rat model of L-DOPA-induced dyskinesia (Miguelez *et al.* 2011). However, the role of noradrenaline and lesions of LC neurons in LID is complex. It seems that the relative degree of damage to LC and substantia nigra pars compacta neurons in combination with chronic L-DOPA treatment and downregulation of alpha2-adrenoceptors contributes to the alterations in LC firing rates (see for references (Miguelez *et al.* 2011) (Fulceri *et al.* 2007) (Ostock *et al.* 2014) (Barnum *et al.* 2012).

Reduced DOPAC and HVA in PD not treated with L-DOPA

In the subgroup of PD patients not treated with L-DOPA (but receiving MAO-B inhibitors and/or DA receptor agonists) we observed reduced CSF levels of L-DOPA, DA, DOPAC and HVA (but not NA and MHPG) as compared to controls. This could reflect the loss of nigrostriatal DAergic neurons. Reductions in DOPAC and HVA may also be due to treatment with MAO-B inhibitors (Parkinson study group 1995). In the present study, five of the seven patients in the PD-N group were also treated with MAO-B inhibitors, and levels of L-DOPA, DOPAC and HVA were not significantly different compared to the two patients only treated with a dopamine D2 receptor agonist. Chronic treatment with a dopamine D2 receptor agonist might increase DA metabolites (Schulte-Herbruggen *et al.* 2012).

In accordance with our findings, Goldstein *et al.* (2012) reported a decrease in CSF L-DOPA levels in untreated PD, but others did not observe significant reductions in L-DOPA in untreated PD (Eldrup *et al.* 1995). The reported reduction

on DA levels has not been shown in other studies (Chia *et al.* 1993; Eldrup *et al.* 1995; Goldstein *et al.* 2012). In some of the PD-N and control samples we could not detect DA because amounts were below detection limit for DA in our HPLC assay (about 10 femtomol on column). For statistical analysis these samples were assigned the lowest detectable level of 0.02 nmol/L. It has been suggested (Goldstein *et al.* 2012) that CSF levels of DA are not a good indicator of central DA deficiency because DA levels are influenced by adaptive changes compensating for the loss of DA neurons or adaptive changes as a result of anti-Parkinsonian medication. In this scenario changes in the CSF levels of the acidic metabolites HVA and DOPAC are better indices of the loss of DA neurons in untreated PD.

Decreased CSF HVA in either drug-naïve PD or after drug-wash-out, has been found by others (Chase and Ng 1972; Davidson *et al.* 1977; Chia *et al.* 1993; Abdo *et al.* 2004; Ishibashi *et al.* 2010), whereas others have not detected a change in CSF HVA levels (Zubenko *et al.* 1986) (LeWitt *et al.* 2011). There is a large inter-individual variation in CSF HVA levels (Parkinson study group 1995) – some of the variation may be due to gradient effects in CSF obtained in the first or later fractions (Dhondt 2004). In our study, the same fraction of CSF was used when comparing control and PD CSF.

DOPAC is considered a more reliable marker for central DA deficiency than HVA because (i) HVA is a secondary metabolite, mainly formed by the successive actions of MAO (possibly within DOPA-decarboxylase containing neurons) and catechol-*O*-methyltransferase (possibly within astrocytes or other cell types) and (ii) DOPAC is produced in DAergic neurons by MAO activity after leakage of DA from synaptic vesicles or following re-uptake of extracellular DA via DAT in the presynaptic membrane (Goldstein *et al.* 2012). Accordingly, decreased DOPAC in CSF of untreated PD has been reported previously (Zubenko *et al.* 1986; Eldrup *et al.* 1995; Goldstein *et al.* 2008, 2012). It is likely that the low levels of DOPAC and HVA in our subgroup of PD patients not treated with L-DOPA are partly due to treatment with MAO-B inhibitors.

Goldstein *et al.* (2012) reported that CSF DOPAC could differentiate PD from controls with 100% sensitivity and 89% specificity in newly diagnosed PD patients. We observed the same sensitivity in our subgroup of PD patients not treated with L-DOPA, with a somewhat lower specificity (Fig. 3a). With regards to sensitivity and specificity, CSF DOPAC performed slightly better than CSF HVA in our study. However, we cannot dismiss that both MAO-B inhibitor and D2 receptor agonist treatment in the PD-N group could affect DOPAC- and HVA-levels.

Age-related changes in monoamine turnover?

Age has several effects on DAergic neurons. The density of tyrosine hydroxylase (TH) positive terminals in striatum gradually decreases (McGeer *et al.* 1977) and the composition of neuromelanine positive cells in substantia nigra pars compacta changes with age, with a significant increase of cells with decreased or no DAT-immunoreactivity (DAT-ir) (Ma *et al.* 1999). It could be speculated that DAT-ir DAergic neurons are more susceptible to age-related neurodegeneration, thus favouring survival of DAergic neurons not expressing DAT. This may explain the higher CSF DA levels and decreased DOPAC/DA ratio (indicating reduced DA turnover) with increasing age in controls. A 6-[^{18}F]-fluoro-L-DOPA PET study found a potentially age-related decrease in vesicular DA storage (Kumakura *et al.* 2010), whereas results from a 6-[^{18}F]-fluoro-L-*m*-tyrosine PET study points to an age-related compensatory increase in AADC activity due to this suboptimal dopaminergic system (Braskie *et al.* 2008). A compensatory increase in DA production combined with decreased vesicular storage capacity could increase CSF DA levels. PET-studies on healthy controls show decreased binding of D2 receptor ligands in the striatum (Wong *et al.* 1984; Volkow *et al.* 1996), which has been interpreted as a downregulation of postsynaptic D2 receptors with age. We would alternatively propose that an increase in CSF DA levels with age could decrease the binding potential of D2 receptor ligands.

In LID we observed much higher CSF DA levels, which seems unlikely to be attributable to age-related changes per se, since LID patients are somewhat younger than PD-L patients.

We also observed an age-dependent decline of the serotonin metabolite 5-HIAA in CSF of controls. There are no reports of an age-related decline of 5-HT neurons (Meltzer *et al.* 1998), thus a decrease in 5-HIAA may also point to decreasing 5-HT turnover with age. None of the controls were diagnosed with severe depression, and none were treated with selective serotonin re-uptake inhibitor or other medication known to alter the CSF 5-HIAA levels. However, motor activity prior to lumbar puncture may affect CSF 5-HIAA levels (Garelis *et al.* 1974).

Study strengths and limitations

Through our spinal tap procedure, quickly processing the CSF, we have minimized the breakdown of catecholamines. Movement disorder specialists made the initial diagnoses and undertook regular follow-up examinations. By allowing patients to continue their L-DOPA-treatment, tapping CSF at a fixed time interval after L-DOPA-intake, we avoid large time-dependent variations in DA CSF levels that are more acutely affected by L-DOPA-intake, whereas the measured DA metabolites reflect the L-DOPA intake of approximately the last 24 h.

The study includes only a small number of PD patients, with minor variation in LID severity in the LID group. The controls were not completely age matched for the PD-N and PD-L groups, and a subgroup of controls had not been

fasting and had the spinal tap performed in supine position, which might affect CSF catecholamine levels.

Conclusion

We investigated the L-DOPA-induced effects on CSF levels of catecholamines and their metabolites in dyskinetic and non-dyskinetic PD patients. In CSF of LID patients we measured much higher DA levels compared to age-matched controls, and a higher DA/L-DOPA ratio, as well as a lower DOPAC/DA ratio compared to non-dyskinetic, L-DOPA-treated patients.

In future longitudinal studies L-DOPA, DA and metabolites would have to be monitored at the beginning of L-DOPA therapy and at various stages of disease progression and treatment before it can be verified that the observed changes in DA and DOPAC/DA ratio in this study can serve as a prognostic marker for LID development.

Acknowledgments and conflict of interest disclosure

This study was funded by the University of Southern Denmark, the Region of Southern Denmark, the Hospital of Southern Jutland, the A.P. Møller Foundation for the Advancement of Medical Science, the Danish Parkinson Association, the Michael J. Fox Foundation, the Memorial Foundation of Knud and Edith Eriksen, the Danske Bank's Foundation, and Else Poulsens Memorial Scholarship. For their assistance in the completion of this study, we thank consultant neurologists Harald Floer, and Matthias Bode, as well as nurses Helle Østerballe, Rikke B. Rosengren, Lene Schroll Kromann, and Dea Meyer. Also, we thank the technicians Ulla Damgaard Munk, Signe Andersen and Dorte Lyholmer, and the Departments of Clinical Biochemistry in Sønderborg, Esbjerg, Odense, and Roskilde Hospitals. Finally, we are grateful to the patients who chose to participate in this project. The authors declare that they have no competing interests.

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APPENDIX III

Paper 3

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2017 | 142 | 756–766 doi: 10.1111/jnc.14104

ORIGINAL ARTICLE

Changes in kynurenine pathway metabolism in Parkinson patients with L-DOPA-induced dyskinesia

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Abstract

L-3,4-Dihydroxyphenylalanine (L-DOPA) is the most effective drug in the symptomatic treatment of Parkinson's disease, but chronic use is associated with L-DOPA-induced dyskinesia in more than half the patients after 10 years of treatment. L-DOPA treatment may affect tryptophan metabolism via the kynurenine pathway. Altered levels of kynurenine metabolites can affect glutamatergic transmission and may play a role in the development of L-DOPA-induced dyskinesia. In this study, we assessed kynurenine metabolites in plasma and cerebrospinal fluid of Parkinson's disease patients and controls. Parkinson patients (n = 26) were clinically assessed for severity of motor symptoms (UPDRS) and L-DOPA-induced dyskinesia (UDysRS). Plasma and cerebrospinal fluid samples were collected after overnight fasting and 1–2 h after intake of L-DOPA or other anti-Parkinson medication. Metabolites were analyzed in plasma and cerebrospinal fluid of controls (n = 14), Parkinson patients receiving no L-DOPA

Received March 22, 2017; revised manuscript received May 30, 2017; accepted June 2, 2017.

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Abbreviations used: 3-HAA, 3-hydroxyanthranilic acid; 3-HAO, 3-Hydroxyanthranilic acid dioxygenase; 3-HK, 3-hydroxykynurenine; 3-OMD, 3-O-methyldopa; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine, serotonin; 5-HTP, 5-hydroxytryptophan; AA, Anthranilic acid; AADC, aromatic amino acid decarboxylase; AMO, anthranilate 3-monooxygenase; CSF, cerebrospinal fluid; DA, dopamine; DLB, lewy body dementia; DOPAC, dihydroxyphenylacetic acid; IDO, indoleamine-2,3-dioxygenase; KATs, kynurenine aminotransferases; KF, kynurenine formamidase; KMO, kynurenine 3-monooxygenase; KYNA, kynurenic acid; KYN, kynurenine; KYNU, kynureninase; LC-MS, liquid chromatography with mass spectrometry; L-DOPA, L-3,4-dihydroxyphenylalanine; LID, levodopa-induced dyskinesia; MAO, monoamine oxidase; MMSE, mini-mental state examination; MOCA, montreal cognitive assessment; NMDA, *N*-methyl-D-aspartate; PD-LID, Parkinson's disease patients receiving L-DOPA, dyskinetic; PD-L, Parkinson's disease patients receiving L-DOPA, non-dyskinetic; PD-N, Parkinson's disease patients not receiving L-DOPA; PD, Parkinson's disease; TDO, tryptophan 2,3-dioxygenase; TRP, tryptophan; UDysRS, unified dyskinesia rating scale; UPDRS, unified Parkinson's disease rating scale; XA, xanthurenic acid.

(n = 8), patients treated with L-DOPA without dyskinesia (n = 8), and patients with L-DOPA-induced dyskinesia (n = 10) using liquid chromatography-mass spectrometry. We observed approximately fourfold increase in the 3-hydroxykynurenine/kynurenic acid ratio in plasma of Parkinson's patients with L-DOPA-induced dyskinesia. Anthranilic acid levels were decreased in plasma and cerebrospinal fluid of this patient group. 5-Hydroxytryptophan levels were twofold increased in all L-DOPA-treated Parkinson's patients. We conclude that a higher 3-hydroxykynurenine/kynurenic acid ratio in plasma may serve as a biomarker for L-DOPA-induced dyskinesia. Longitudinal studies including larger patients cohorts are needed to verify whether the changes observed here may serve as a prognostic marker for L-DOPA-induced dyskinesia.

Keywords: 3-hydroxykynurenine, biomarkers, kynurenic acid, levodopa, metabolomics, tryptophan.
J. Neurochem. (2017) **142**, 756–766.

The most effective drug in the symptomatic treatment of Parkinson's disease (PD) is L-DOPA (L-3,4-dihydroxyphenylalanine), but chronic use is associated with serious motor complications, L-DOPA-induced dyskinesia (LID). LID is observed in more than half the patients after 10 years of treatment (Schapira *et al.* 2009). Risk factors for early onset LID include younger age, more severe disease at baseline, and higher daily dose of L-DOPA (Rascol *et al.* 2006; Cheshire *et al.* 2014), but biomarkers to predict the risk of motor complications are not yet available.

Only a few studies have used metabolomic approaches to find biomarkers for PD in plasma (Bogdanov *et al.* 2008; Johansen *et al.* 2009; Roede *et al.* 2013; Trupp *et al.* 2014; Hatano *et al.* 2016; Wuolikainen *et al.* 2016) or cerebrospinal fluid (CSF) (Lewitt *et al.* 2013; Trupp *et al.* 2014; Wuolikainen *et al.* 2016), but these studies have not addressed the metabolic effects of L-DOPA treatment and its long-term consequences for the development of LID. The above-mentioned metabolomic studies of blood and CSF suggested alterations in purine, tyrosine and tryptophan (TRP) metabolism in PD (Ascherio *et al.* 2009; Johansen *et al.* 2009; Lewitt *et al.* 2013; Trupp *et al.* 2014; Hatano *et al.* 2016), including increased 3-hydroxykynurenine/kynurenic acid (3-HK/KYNA) ratios in CSF from demised patients (Lewitt *et al.* 2013). Most recently, a comprehensive urinary metabolic profile was reported that identified unique metabolic markers for idiopathic PD associated with the progression of disease (Luan *et al.* 2015a,b), including TRP metabolites.

L-DOPA treatment has a major impact on tyrosine metabolism (De Deurwaerdere *et al.* 2017), but the effect of L-DOPA on TRP metabolism and its possible role in the development of L-DOPA-induced dyskinesia has not been clarified. TRP is not only a precursor of serotonin (5-HT), but also for kynurenine (KYN), which can be degraded to several other neuroactive compounds, including KYNA (neuroprotective, antagonist of the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor), 3-HK (neurotoxic, generates free radicals), and quinolinic acid (neurotoxic, NMDA receptor agonist) (Schwarcz *et al.* 2012). The KYN pathway (see Fig. 1) is the most important route of TRP degradation in humans.

Both pre-synaptic and post-synaptic maladaptive changes contribute to the pathophysiology of LID (Bastide *et al.* 2015; De Deurwaerdere *et al.* 2017). Recently, we reported elevated dopamine (DA)/L-DOPA and reduced dihydroxyphenyl acetic acid/DA ratios in CSF of LID patients as compared to non-dyskinetic PD patients receiving L-DOPA, suggesting increased DA release from non-DA cells and deficient DA re-uptake in PD-LID patients (Andersen *et al.* 2017). In addition to uncontrolled dopamine release acting on supersensitive D1 receptors, enhanced glutamatergic signaling may contribute to LID. Experimental studies have shown that a change in the ratio of kynurenine metabolites can alter glutamatergic signaling and may protect against excitotoxicity mediated via NMDA receptors. KYN metabolism can be manipulated *in vivo* using tryptophan-2,3-dioxygenase or kynurenine 3-monooxygenase (KMO) inhibitors (Campesan *et al.* 2011; Breda *et al.* 2016). Interestingly, prolonged systemic administration of the KMO inhibitor Ro 61-8048, which blocks 3-HK synthesis and increases KYNA levels in the brain, reduces LID in Parkinsonian, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys (Gregoire *et al.* 2008).

Here, we investigated whether KYN metabolites in plasma or CSF are altered in PD patients as compared to controls and whether altered ratios of KYN metabolites are associated with L-DOPA use or LID.

Methods

PD patients and controls were recruited from different neurological clinics in the Region of Southern Denmark (Hospitals in Sønderborg and Odense), a private neurological practice (Esbjerg) and a neurological clinic in Region Zealand (Roskilde). Twenty-five PD patients and one Lewy body dementia (DLB) patient (details described under 'subgroups of PD patients') were included, all diagnosed by movement disorder specialists using the UK Brain Bank Criteria for idiopathic PD (20). Control patients were referred by their physician or practicing neurologist for further neurological examination including lumbar puncture. Neither controls nor PD patients were receiving special diets, which may affect kynurenine

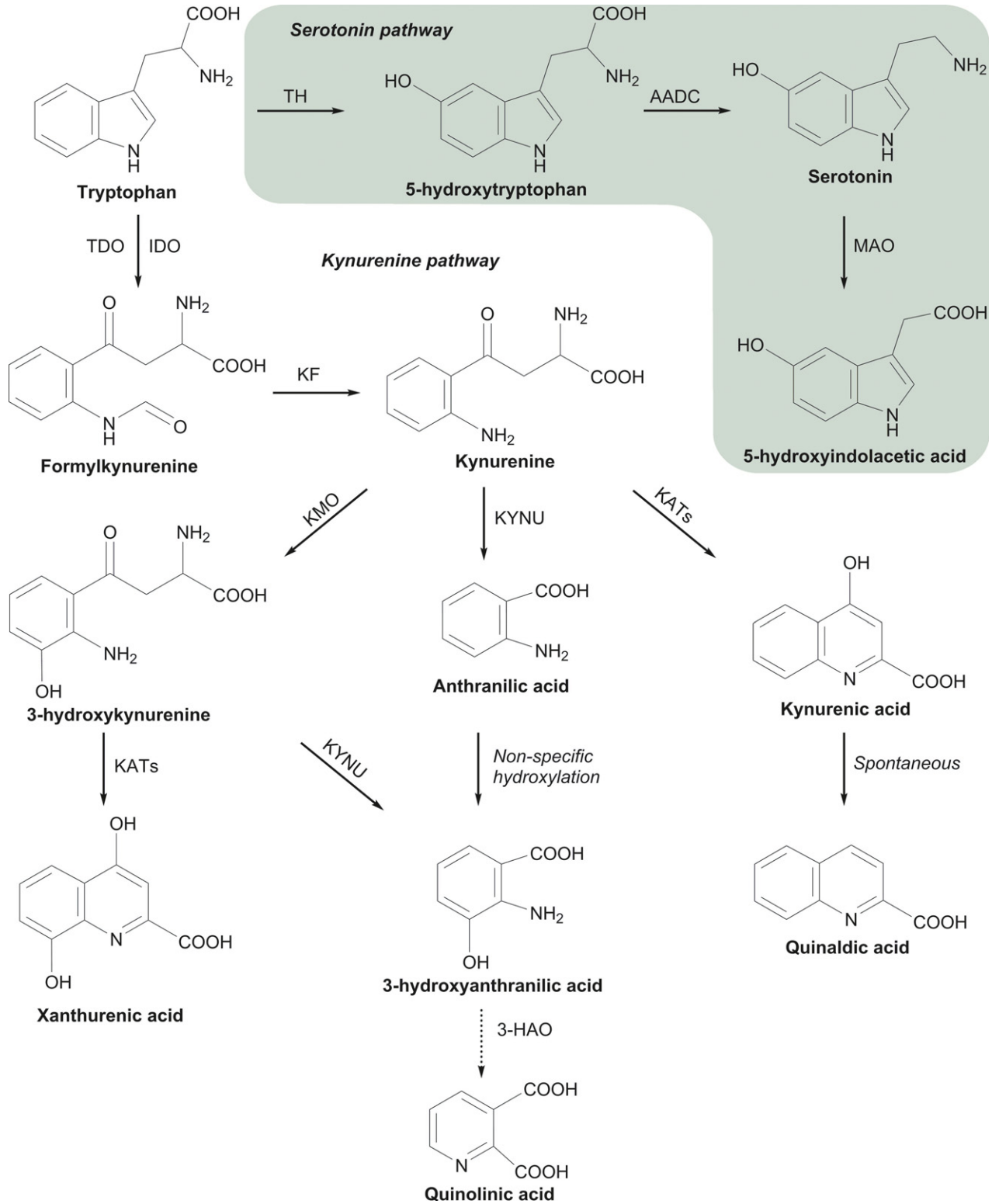


Fig. 1 Kynurenine and serotonin pathway of tryptophan (TRP) metabolism. TDO: tryptophan 2,3-dioxygenase; IDO: indoleamine-2,3-dioxygenase; KF: kynurenine formamidase; KMO: kynurenine 3-monooxygenase; KYNU: kynureninase; KATs: kynurenine aminotransferases; 3-HAO: 3-hydroxyanthranilic acid dioxygenase; AMO: anthranilate 3-monooxygenase; TH: tryptophan hydroxylase; AADC: aromatic amino acid decarboxylase; MAO: monoamine oxidase.

pathway metabolism. In Table 1, demographic and clinical data are summarized for controls and PD patients providing plasma samples. In Table S1, patient data are summarized for CSF samples. Further clinical details of patients and controls can be found in Andersen *et al.* (2017). All PD patients and controls were informed in writing and orally about the project, and gave written consent prior to participation. The project was approved by the local ethics committee of the Region of Southern Denmark (S-20130098). The study conforms to The Code of Ethics of the World Medical Association.

CSF sampling

Lumbar punctures of PD patients were performed between 09.30 and 10.00 am after overnight fasting. Patients on L-DOPA were instructed to (when possible) take their morning dose at 08.00 am, but time of intake varied from 05.30 am to 08.00 am. The procedure was performed in a sitting position, using an atraumatic Pencan® (B.Braun, Frederiksberg, Denmark) (0.53 × 88 mm) needle at the L3/L4 level. The CSF was centrifuged at 4°C for 10 min at 2000 g. The supernatant was aliquoted into Sarstedt® (Sarstedt, Nümbrecht, Germany) tubes and placed on dry ice immediately until storage at −80°C.

For controls, the procedure was performed between 09.30 am and 12.15 pm using an atraumatic Pencan® (B.Braun, Frederiksberg, Denmark) (0.53 × 88 mm) needle at the same lumbar level. A subgroup of controls had not been fasting before withdrawal of CSF. Five out of 14 control patients had the procedure done in a sitting position without measurement of the CSF pressure, whereas nine had the procedure done lying on the side with CSF pressure measurement before withdrawing CSF as part of their investigation. Aside from this the same steps for sampling CSF were followed as described above. CSF samples were obtained from 14 controls and 22 PD patients.

Blood sampling

Blood was drawn from the cubital fossa immediately after the lumbar puncture in both patients and controls. Four BD Vacutainer™ citrate tubes (Becton Dickinson A/S, Kgs.Lyngby, Denmark) were filled and gently turned four times to prevent coagulation. Within 30 min the four tubes were centrifuged at room temperature for 10 min at 1800 g. The plasma supernatant was evenly distributed into two 15 mL tubes and centrifuged at room temperature for 10 min at 3000 g. The supernatant was then aliquoted into Saarstedt® tubes and put on dry ice until storage at −80°C. Plasma samples were obtained from 14 controls and 26 PD patients.

Clinical rating

Clinical rating was performed on the same day as the LP procedure. PD motor symptom severity was rated in the ON-stage using the MDS-UPDRS part III (Goetz *et al.* 2007). For patients using L-DOPA, this score and the unified dyskinesia rating scale (UDysRS) objective score procedure (Goetz *et al.* 2008) was performed approximately 50 min after drug intake. The procedures were recorded on video for later assessment. Cognitive rating was done using the mini-mental state examination (MMSE) and Montreal cognitive assessment (MOCA) scales.

Subgroups of PD patients

PD patients were divided into three groups: patients not receiving L-DOPA (PD-N, $n = 8$), patients receiving L-DOPA but without dyskinesia (PD-L, $n = 8$), and patients receiving L-DOPA and having dyskinesia (PD-LID, $n = 10$). A DLB patient with bradykinesia and a dopamine transporter-scan with marked bilateral decreased FP-CIT-uptake in the basal ganglia was included in the PD-L group, since this patient was treated with L-DOPA and was not dyskinetic. See Table 1 (plasma samples) and Table S1 (CSF samples) for demographic and clinical details. Patients assigned to PD-LID either had a positive UDysRS objective score ($n = 8$), or – these patients were recruited before the use of the UDysRS – clearly dyskinetic presentation ($n = 1$), or amnestic description of LID symptoms ($n = 1$). One patient in

Table 1 Plasma samples – Demographic and clinical data

	Control	PD-N	PD-L	PD-LID
Cases (*n*)	14	8	8	10
Age (years)	55.8 ± 11.6	62.6 ± 6.6	67.4 ± 11.8[a]	59.9 ± 7.9
Sex (F/M)	6/8	3/5	0/8	3/7
UPDRS III	–	18.8 ± 6.8	36.6 ± 7.5	30.1 ± 11.8
UDysRS objective score	–	0	0	7.8 ± 5.7
Symptom duration (years)	–	3.9 ± 2.4	6.6 ± 3.7	8.8 ± 4.2
LED (mg/day)*	10 ± 40	293 ± 126	663 ± 416	857 ± 288
LED-DOPA only (mg/day)	–	–	439 ± 329	593 ± 248
MAO inhibitors	0/14	5/8	1/8	3/10
DA receptor agonists	1/14	7/8	5/8	8/10
MOCA	–	28 ± 2.0	26 ± 2.5	26 ± 1.6
MMSE	–	29 ± 1.4	28 ± 3.7	29 ± 0.7

*LED, levodopa-equivalent dose; converts the dose of any anti-parkinsonian treatment into the equivalent dose of immediate release L-DOPA. PD-N, Parkinson's disease not receiving L-DOPA; PD-L, Parkinson's disease receiving L-DOPA, non-dyskinetic; PD-LID, Parkinson's disease receiving L-DOPA, dyskinetic; L-DOPA, Levodopa; F, female; M, male; UPDRS, Unified Parkinson's disease rating scale; UDysRS, Unified dyskinesia rating scale; MOCA, Montreal cognitive assessment; MMSE, Mini-mental state examination; Numerical values are mean ± SD. [a]PD-L older than controls ($p < 0.01$), but not significantly different from PD-N and PD-LID.

PD-L and one patient in PD-LID received anti-depressant medication.

Blinding of samples

During LC-MS processing (see below) of plasma and CSF samples, samples were labeled with C for controls or P for patients, but it was not known whether patients were assigned to the PD-N, PD-L, or PD-LID subgroups.

Sample preparation

One hundred microliters of plasma and CSF, respectively, were thawed on ice for 1 h before addition of 350 μL 85% methanol including heavy labeled internal standards (0.05 μM $^{13}C_2{}^{15}N$-3-hydroxykynurenine (Biozol, TRC-H943697), 0.05 μM $^{13}C_6$-kynurenic acid (PerkinElmer, CUSN83060000EA), 2 μM D_6-kynurenine (Cambridge isotope Laboratories, DLM-7842), and 2 μM mixture of 17 heavy isotope-labeled amino acids (Cambridge isotope Laboratories, MSK-A2-1.2). The samples were vortexed for 30 s and left at −20°C for 30 min before centrifugation at 16 000 g for 20 min. A quantity of 110 and 120 μL of supernatants from plasma and CSF, respectively, were transferred to new tubes prior to lyophilization. The lyophilized samples were resuspended in 20 μL 1% formic acid (FA).

LC–MS and data analysis

Ten microliters of sample was injected using a 1290 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax Eclipse Plus C18 guard column (2.1 × 50 mm and 1.8 μm particle size) and an analytical column (2.1 × 150 mm and 1.8 μm particle size) kept at 40°C. The compounds were eluted using a flow rate of 300 μL/min and the following composition of A (0.1% FA) and B (0.1% FA, acetonitrile) solvents: 97% A from 0 to 5 min, 97–85% A from 5 to 8 min, and 85-60% from 8 to 18 min before equilibration for 3 min with the initial conditions. Eluting metabolites were detected by a 6530 quadrupole time of flight mass spectrometer (Agilent Technologies) operated in positive ion mode scanning from 100 to 1000 m/z with the following settings: Gas temp at 300°C, drying gas at 8 L/min, nebulizer at 35 psig, sheath gas temp at 350°C, sheath gas flow at 11 min/l, VCap at 3500 V, fragmentor at 125 V, and skimmer at 65 V. Each spectrum was internally calibrated during analysis using the signals of purine (121.0509) and Hexakis 1H,1H,3H-tetrafluoropropoxy phosphazine (922.0098), which was delivered to a second needle in the ion source by an isocratic pump running with a flow of 20 μL/min.

A library with retention times obtained from synthetic standards (not available for N-formyl kynurenine), and exact mass of the metabolites of interest was constructed using MassHunter PCDL Manager v. B.07.00 (Agilent Technologies). Chromatograms for all compounds were extracted and quantified using Profinder v. B.08.00 (Agilent Technologies) with a mass tolerance of 20 ppm and retention time tolerance of 0.1 min. Chromatograms were subsequently manually validated. In-source-induced diagnostic fragments were used for validation of the identification (Table S2). A chromatogram of a plasma sample is shown in Figure S1. Quantitative linearity for all compounds (except N-formyl kynurenine) was tested using synthetic standards in different amounts covering the signal range of the measured endogenous metabolites (Figure S4).

Statistics

Statistical differences in metabolites or ratios among controls and PD subgroups were analyzed by non-parametric ANOVA (Kruskall–Wallis test) and shown by one or more asterisks following the compound name in the bar charts. Subsequently, PD-LID values were compared to controls and the other PD groups using Dunn's multiple comparison test. Statistical differences were calculated with and without inclusion of the DLB patient in the PD-L group. Significant differences are indicated in the bar charts, where * denotes significance levels of $p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, and $^{****}p < 0.00005$.

Results

LC-MS was used to quantify TRP metabolites along the KYN and serotonin pathways. The levels of KYN and three of its major metabolites 3-HK, KYNA, and anthranilic acid (AA) in plasma and CSF of controls and the three PD groups are shown in Fig. 2. Absolute quantification was done for KYN, 3-HK, and KYNA as heavy isotope-labeled versions of these were added during the extraction. AA and other metabolite levels (Figure S2) were expressed as relative abundances.

Plasma KYN levels (low μM range) are about a factor 50 higher than CSF levels, whereas 3-HK and KYNA levels (nM range) are 15–20-fold higher in plasma as compared to CSF. AA levels in plasma and CSF could not directly be compared because no internal, heavy isotope-labeled standard was used. The results for these metabolites showed significant decreases for KYNA and AA in plasma of PD-LID patients. A similar trend was observed for these metabolites in CSF of PD-LID, but only the decrease in AA reached significance as compared to PD-L (with and without inclusion of the DLB patient).

Among controls, only two out of eight males and three out of six females had been fasting overnight before withdrawal of plasma or CSF. In plasma of the small non-fasting group, mean values for most metabolites were generally somewhat higher, but these differences did not reach statistical significance. Ratios of KYN metabolites were not affected by

Fig. 2 Plasma and cerebrospinal fluid (CSF) levels of kynurenine (a), 3-hydroxykynurenine (b), kynurenic acid (c), and anthranilic acid (d). Anthranilic acid was quantified relatively as no internal standard of the compound was used. Patient groups and statistics are described in material and methods. Abbreviations of patient groups: see legend to Table 1. ANOVA (Kruskall–Wallis test) for plasma 3-hydroxykynurenine (b) and plasma and CSF anthranilic acid (d) revealed significant changes among groups. ANOVA (Kruskall–Wallis test) for plasma kynurenic acid (c) did not reach significance ($p = 0.08$), but subsequent Dunn's test revealed significant reduction in PD-LID as compared to PD-L. Data are means ± standard error of mean (SEM).

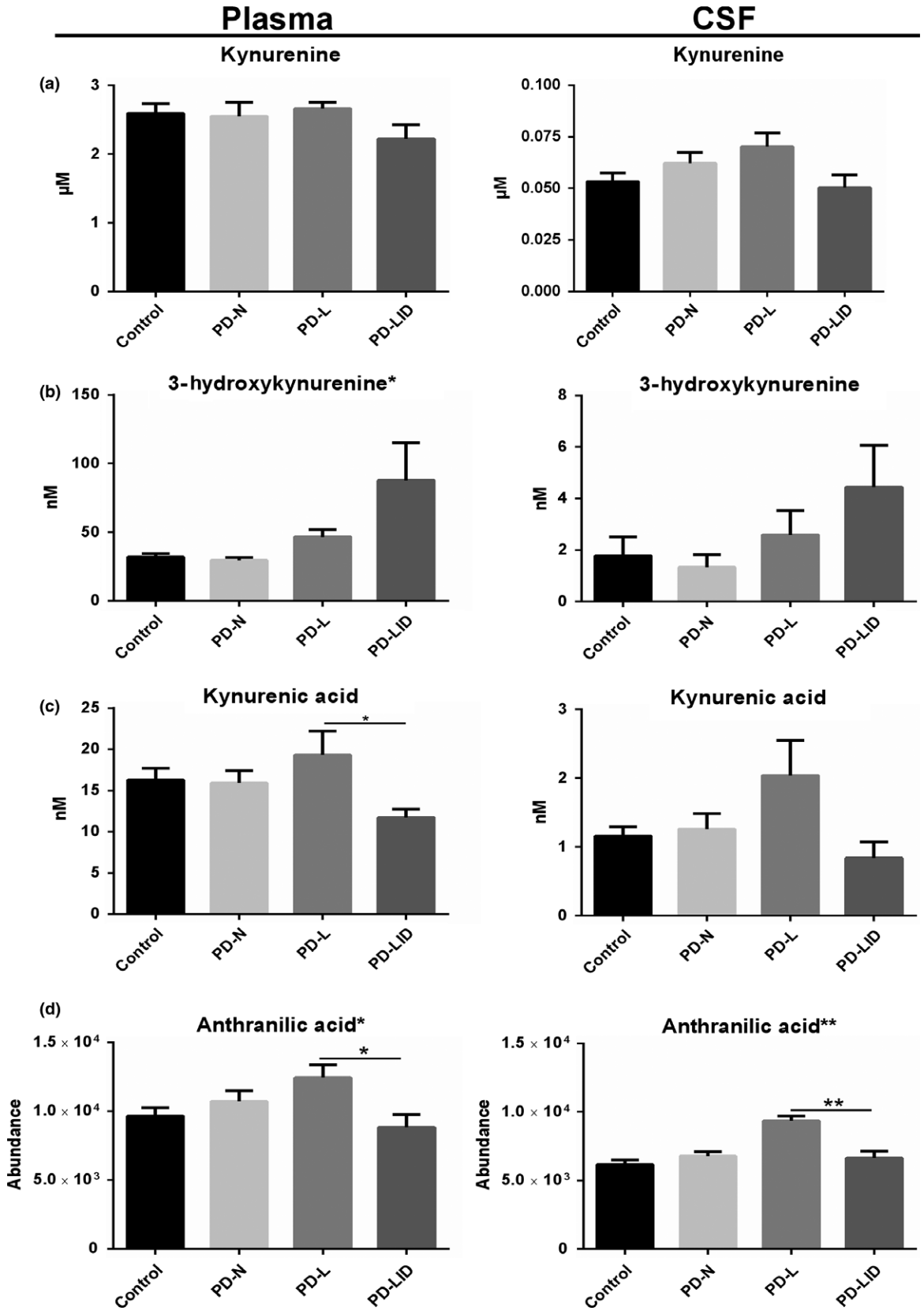

Plasma
CSF
Kynurenine
Kynurenine
(a)
μM
μM
Control
PD-N
PD-L
PD-LID
3-hydroxykynurenine*
3-hydroxykynurenine
(b)
nM
nM
Kynurenic acid
Kynurenic acid
(c)
nM
nM
*
Anthranilic acid*
Anthranilic acid**
(d)
Abundance
Abundance
*
**

Fig. 3 Ratios of selected metabolites in plasma and cerebrospinal fluid (CSF). The 3-HK/KYNA ratio (a) demonstrates the relative activity between KMO and KATs. The 3-HK/KYN ratio (b) indicates KMO activity, the 3-HAA/3-HK ratio (c) indicates KYNU activity, and XA/3-HK ratio (d) indicates activity of KATs. Data are means ± SEM. Abbreviations of patient groups: see legend to Table 1. Abbreviations used for metabolites and enzymes: 3-HK: 3-hydroxykynurenine; 3-HAA: 3-hydroxyanthranilic acid; KATs: kynurenine aminotransferases; KMO: kynurenine 3-monooxygenase; KYN: kynurenine ;KYNA: kynurenic acid; KYNU: kynureninase; XA: xanthurenic acid.

fasting status. Therefore, fasting and non-fasting controls were pooled for comparison with PD subgroups.

Figure 3 shows the ratios for these and other KYN metabolites in plasma and CSF. The data indicate alterations between the different branches of KYN pathway metabolism in PD and PD-LID. In plasma, we observed significant, highly elevated 3-HK/KYNA and 3-HK/KYN ratios and significantly decreased 3-hydroxyanthranilic acid/3-HK and xanthurenic acid/3-HK ratios in PD-LID patients as compared to controls or PD-N. Excluding the two PD patients receiving anti-depressants (one in PD-L and one in PD-LID) did not alter the significant findings in plasma.

In plasma of controls, we observed significantly higher levels (*t*-test, $p < 0.05$) of some metabolites in males as compared to females: tryptophan, kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, and xanthurenic acid. However, the observed male-female differences in plasma of controls cannot account for the observed differences in 3-HK/KYNA ratio between PD-LID (3 females, 7 males) and controls (6 females, 8 males) or for the observed difference in anthranilic acid between PD-L (0 females, 8 males) and PD-LID (3 females, 7 males).

Other metabolite ratios in plasma are shown in Figure S2, with a significantly reduced KYN/TRP ratio in PD-LID as compared to PD-L and no significant differences in KYNA/KYN and AA/KYN ratios.

In CSF, we observed a similar trend with a highly elevated mean of 3HK/KYNA in PD-LID, but because of large variation and lower n (Table S1), these changes did not reach significance. In CSF of controls, no effect of fasting status or gender was observed for any of the metabolites.

In PD patients (all groups), plasma and CSF levels of KYN ($r^2 = 0.21$, $p = 0.03$) and 3-HK ($r^2 = 0.26$, $p = 0.02$) were significantly correlated, but plasma and CSF levels of KYNA were not correlated.

We assessed also L-DOPA, 3-O-methyldopa (3-OMD), and TRP metabolites along the serotonin pathway (Figure S2 and S3). As expected, the L-DOPA-treated PD patients (PD-L and PD-LID) showed highly elevated L-DOPA and 3-OMD levels in plasma and CSF. TRP levels in plasma and CSF were not different between controls and the three PD groups, but 5-hydroxytryptophan (5-HTP) was about twofold increased in PD-L and PD-LID.

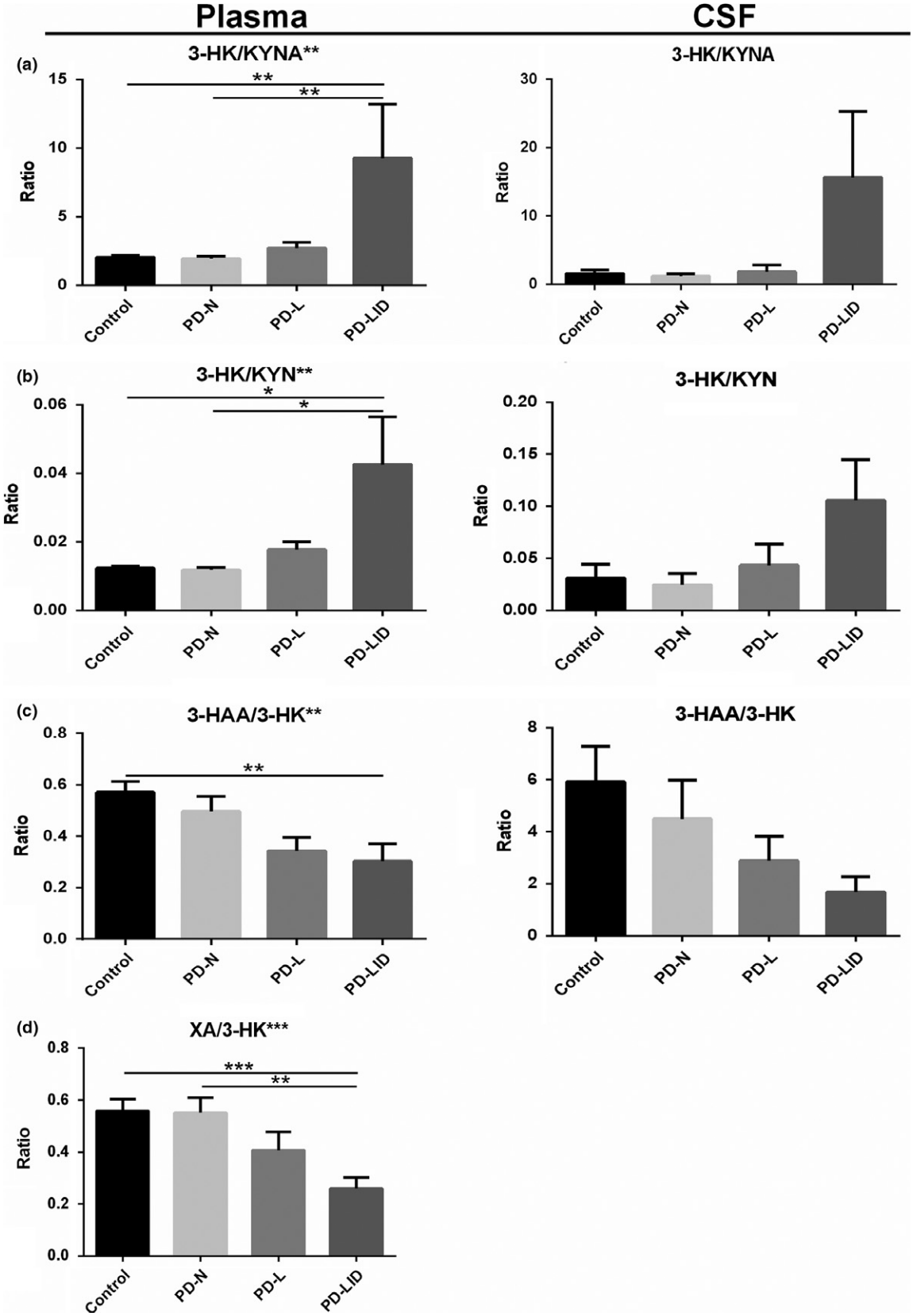
In conclusion, we observed significant changes in KYN metabolism in PD-LID, differentiating this group not only from controls, but also from non-dyskinetic PD, that is, PD-N and PD-L.

Discussion

Here, we present evidence of an altered KYN pathway metabolism in plasma and CSF of PD patients with L-DOPA-induced dyskinesia. The overall change of KYN metabolism in PD-LID is a shift toward the production of 3-HK (Fig. 4). The major findings are the significant decreases of AA and KYNA in plasma of dyskinetic (PD-LID) versus non-dyskinetic L-DOPA-treated PD patients (PD-L) and a significant increase of the 3-HK/KYNA ratio in plasma of PD-LID versus controls and PD-N. The presented quantitative LC-MS analysis of tryptophan and kynurenine metabolites in human plasma and CSF showed absolute levels of the measured analytes in agreement with those reported previously for plasma (Widner *et al.* 2002; Forrest *et al.* 2010; Savitz *et al.* 2015) and CSF (Olsson *et al.* 2012; Kegel *et al.* 2014; de Bie *et al.* 2016; Sellgren *et al.* 2016).

The 3-HK/KYNA ratio reflects the balance between the neurotoxic and neuroprotective metabolites. An increase of this ratio may indicate increased activity of KMO in relation to kynurenine aminotransferases and kynureninase activities (see Fig. 1). An increased 3-HK/KYN and decreased 3HAA/3-HK and XA/3-HK ratios in PD-LID plasma (Fig. 3) are also in line with increased KMO activity. We did not observe significant changes in tryptophan or KYN levels in plasma or CSF, indicating that kynurenine metabolism in PD-LID is shifted toward 3-HK production – catalyzed by KMO – and a down-regulation of KYNA (via kynurenine aminotransferases) and AA (via kynureninase) synthesis. These changes were not observed in the two subgroups of non-dyskinetic PD patients, indicating that the three- to fourfold increase of 3HK/KYNA ratio in plasma or CSF may be considered a specific biomarker of LID.

The enhanced 3HK/KYNA ratio in plasma and a similar tendency in CSF suggest that a similar change in KYN metabolites occurs in the basal ganglia at the synaptic level, where it may give rise to increased glutamatergic transmission, which may facilitate LID. The rationale for this is that lower KYNA levels allow endogenous glycine to potentiate NMDA receptor function and pre-synaptic nicotinic acetylcholine receptors on glutamatergic terminals are more easily activated (Schwarcz *et al.* 2012). In PD spine loss occurs on the dendrites of medium spiny striatal neurons containing dopamine D2 receptors (Neely *et al.* 2007). This selective spine loss may contribute to the development of LID (Schuster *et al.* 2009). Increased levels of 3-HK, which increase oxidative stress (Okuda et al.1998), and decreased levels of KYNA, which


Plasma
CSF
(a)
3-HK/KYNA**
3-HK/KYNA
Ratio
15
10
5
0
30
20
10
0
**
**
Control
PD-N
PD-L
PD-LID
(b)
3-HK/KYN**
3-HK/KYN
0.06
0.04
0.02
0.00
0.20
0.15
0.10
0.05
0.00
*
*
(c)
3-HAA/3-HK**
3-HAA/3-HK
0.8
0.6
0.4
0.2
0.0
8
6
4
2
0
(d)
XA/3-HK***
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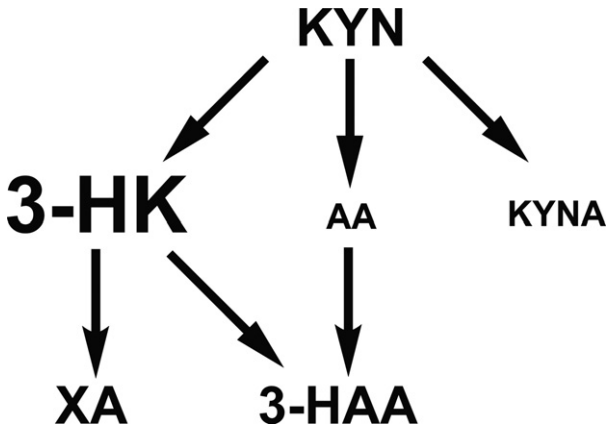

Fig. 4 Diagram of major changes in KYN metabolism in LID. Size of letters indicate changes in metabolite levels as compared to controls and non-dyskinetic PD.

increase and vulnerability for excitoxicity (Amori *et al.* 2009), may contribute to striatal spine loss and the development of LID.

In the MPTP monkey model of PD, LID symptoms can be reduced by enhancing brain KYNA production using systemic treatment with an inhibitor of kynurenine 3-monooxygenase (KMO) (Gregoire *et al.* 2008). In addition, systemic inhibition of KMO shows marked neuroprotective effects in a *Drosophila* model of Huntington's disease (Campesan *et al.* 2011). Provided that future studies confirm the present results of increased 3HK/KYNA ratios in dyskinetic PD patients, clinical intervention studies using KMO inhibitors should be considered to alleviate LID symptoms and to confirm the pathophysiological role of kynurenine metabolites in LID.

Other PD biomarker studies targeting TRP and KYN metabolites have shown increases in KYN/TRP ratio in plasma (Widner *et al.* 2002) and increases in 3HK/KYNA ratio in postmortem obtained CSF (Lewitt *et al.* 2013). Increases of 3-HK and decreases of KYNA have been reported previously in brain tissue samples (frontal cortex, putamen, substantia nigra) of PD patients with the largest changes observed in PD patients treated with L-DOPA (Ogawa *et al.* 1992). These previous studies did not address eventual differences between L-DOPA-treated PD patients with or without LID. Despite the small sample sizes of our PD subgroups, our study clearly shows that KYN pathway metabolism is changed in PD-LID with a decreased KYN/TRP ratio and an increased 3HK/KYNA ratio, which was not present in PD-L or PD-N.

The PD-LID group is not only characterized by the presence of LID, but also by a younger age of onset and a longer duration of disease (see Table 1). Some of these factors, for example, more advanced PD, increased oxidative stress, increased inflammation, may contribute to the observed changes in KYN metabolism (Lim *et al.* 2017).

TRP is not only degraded via the kynurenine pathway, it is also the precursor of 5-HTP and 5-HT, reactions catalyzed by TRP hydroxylase and amino acid decarboxylase. In plasma we observed about twofold increases of 5-HTP in PD-L and PD-LID as compared to controls and PD-N. This effect can be explained by the co-treatment with peripheral decarboxylase inhibitors and also competition between L-DOPA and 5-HTP, which are both substrates for aromatic amino acid decarboxylase.

In agreement with a higher daily L-DOPA intake in PD-LID patients (Table 1), we observed slightly higher L-DOPA and 3-OMD levels in PD-LID, although not significantly different from PD-L. 3-OMD levels are much higher than L-DOPA, because it has a much longer half-life than L-DOPA (Tohgi *et al.* 1995). L-DOPA and 3-OMD are, like KYN, transported into the brain via the large neutral amino acid transporter and may inhibit the transport of KYN over the blood-brain barrier and into astrocytes (Asanuma and Miyazaki 2016; Sekine *et al.* 2016). A lower availability of KYN in astrocytes in PD-LID, may reduce central KYNA production. In contrast to cerebral 3-HK, which can be derived from the circulation or from production in brain microglia or macrophages, cerebral KYNA levels are almost entirely dependent on synthesis in brain astrocytes (Fukui *et al.* 1991; Pardridge 1998).

Changes in KYN metabolites have been observed in other neurological and psychiatric disorders, including, Huntington's disease (Forrest *et al.* 2010; Byrne and Wild 2016), schizophrenia (Forrest *et al.* 2010; Chiappelli *et al.* 2014; Kegel *et al.* 2014; Fazio *et al.* 2015; Oxenkrug *et al.* 2016) bipolar disorder, (Olsson *et al.* 2012; Lavebratt *et al.* 2014; Savitz *et al.* 2015; Sellgren *et al.* 2016) and other brain disorders (Vecsei *et al.* 2013; Lovelace *et al.* 2016; Watzlawik *et al.* 2016). In schizophrenia and bipolar disorder *increased* levels of KYNA have been observed in saliva, CSF and postmortem brain tissue, whereas in Huntington's disease decreased KYNA and increased 3-HK and quinolinic acid has been observed in CSF and postmortem brain tissue. TRP metabolism is also influenced by gut microbiota with significant consequences for CNS function and dysfunction (Kennedy *et al.* 2017). Thus it can be speculated that differences in gut microbiota among PD patients play a role in the response to L-DOPA therapy and the risk of developing LID.

We conclude that our present LC-MS methodology to detect KYN metabolites in body fluids provides a unique tool to monitor L-DOPA-treated PD patients for increasing risk of LID. Further validation of the 3HK/KYNA ratio as a biomarker of LID in plasma is needed in larger patient cohorts and longitudinal studies, including multiple samples from the same patients obtained before and after development of LID.

Acknowledgments and conflict of interest disclosure

We thank consultant neurologists Harald Floer, Akram Kamal, Helle Thagesen and Troels W. Kjær and nurses Helle Østerballe, Rikke B.

Rosengren, Lene Schroll Kromann, and Dea Meyer for assistance in the completion of this study. Also thanks to the Departments of Clinical Biochemistry in Sønderborg, Esbjerg, Odense, and Roskilde Hospitals. We are grateful to the patients who chose to take part in this project. This study was supported by the Michael J Fox Foundation for Parkinson's Disease Research (grant ID 10241 to JBG), the Danish Parkinson Society (grant 2015 to JBG), Jaschafonden (grant 2014 to JBG), Villum fonden (grant VKR023179), and Region of Southern Denmark, SDU Health Sciences Faculty and Hospital Southern Jutland (co-financed Ph.D. stipend to ADA), the A.P.Møller Foundation for the Advancement of Medical Science (grant to ADA), the Memorial Foundation of Knud and Edith Eriksen (grant to ADA), the Danske Bank's Foundation (grant to ADA), and Else Poulsens Memorial Scholarship (grant to ADA). The authors have no conflicts of interest or financial disclosures to declare.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Elution profile of compounds of interest.

Figure S2. Levels and ratios of selected plasma metabolites.

Figure S3. Levels and ratios of selected CSF metabolites.

Figure S4. Linearity curves of measured compounds.

Table S1. CSF samples - Demographic and clinical data.

Table S2. Identified compounds.

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APPENDIX IV

Manuscript 4

Title

L-Tryptophanol for the quantification of prefibrillary proteins in CSF and plasma in Parkinson's disease

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Abstract

Background:

Parkinson's disease (PD) is a common neurodegenerative disease characterized by motor symptoms like bradykinesia, rigidity and resting tremor as well as the accumulation of Lewy Bodies, especially in the substantia nigra, pars compacta. Misdiagnosis is common in the early stages of the disease, in particular before the occurrence of motor symptoms. A better understanding of the pathophysiological mechanisms behind PD and an early diagnostic and prognostic marker is warranted. Prefibrillary amyloid formation may play a role in the detrimental processes occurring in neurons in PD. The substance L-Tryptophanol is fluorescent and this fluorescence can be quenched by the presence of prefibrillary amyloids.

Objective:

To quantify the amount of prefibrillary amyloid proteins in cerebrospinal fluid (CSF) and blood plasma of patients and controls.

Methods:

26 PD patients and 15 controls were included. Lumbar punctures and blood samples were drawn in the morning. The same day PD patients were clinically rated using the Unified Parkinson's Disease Rating Scale, the Unified Dyskinesia Rating Scale, the Montreal Cognitive Assessment and the Mini-Mental State Examination. PD patients were divided into three groups: PD-N; not receiving L-DOPA, PD-L; receiving L-DOPA, not dyskinetic, PD-LID; receiving L-DOPA, dyskinetic.

CSF and blood samples were analysed using an L-Tryptophanol fluorescence assay, providing a Trol score corresponding to the load of prefibrillary amyloids.

Results:

In PD, CSF Trol was significantly lower compared to controls, especially in L-DOPA treated patients. Reversely, plasma Trol was significantly higher in PD. The plasma Trol/CSF Trol ratio even more significantly separated PD from controls. PD patients had higher CSF total protein concentrations, and the CSF Trol/CSF total protein concentration significantly differed all PD groups from the control group.

Conclusion:

The L-Tryptophanol fluorescence assay could potentially be used as a diagnostic tool for PD. Larger studies, also including atypical PD, are warranted.

Background

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease(1). PD patients develop the characteristic motor symptoms of bradykinesia, rigidity, and/or resting tremor (2) when 30% or more of nigrostriatal dopaminergic neurons are lost(3). Neurodegeneration has thus progressed for potentially many years when patients are diagnosed as having PD. Premotor symptoms of PD include hyposmia, depression, REM-sleep behaviour disorder (RSBD) and constipation(4) (for review see (5)). In general premotor symptoms are not specific indicators of PD.

No disease-modifying treatment exists, slowing down the neurodegenerative processes in PD. The best symptomatic treatment is provided by using levodopa (L-DOPA). However, after some years of treatment (or at more advanced stages of PD), L-DOPA will gradually lose its therapeutic effect, resulting in marked fluctuations of PD motor symptoms and increasing risk of developing debilitating side effects such as L-DOPA-induced dyskinesia (LID)(6).

The presence of Lewy bodies in specific brain areas is the neuropathological hallmark of PD(7). Braak et al proposed a neuropathological staging of the disease correlating the degree of LB pathology with the clinical Hoehn & Yahr score (8). Alpha-synuclein (α-syn) is the main constituent of LB(9), and point mutations in the α-syn gene (10) are related to autosomal-dominant genetic forms of PD. A point mutation caused α-syn to form beta-sheet structures that are prone to aggregation. The α-syn protein is prone to aggregation, and especially aggregated oligomeric types of α-syn (o-α-syn) play a significant role in the disruption of normal cellular function and subsequent cell death in PD(11). O-α-syn can easily spread from neuron to neuron through exosomal transmission(12), and this propagation of toxic proteins has fuelled the hypothesis of PD being a prion like disorder(13).

The need for earlier and more precise diagnostic procedures for PD has resulted in an extensive research into potential biomarkers. A review of the current literature has shown that results from measuring total α-syn (t-α-syn) in the cerebrospinal fluid (CSF) are divergent(14). Although some research has been done into modified versions of α-syn, such as phosphorylated versions that aggregate more easily, o-α-syn has not been studied as intensively. Some of the studies using modified versions of enzyme linked immunosorbent assays (ELISA) have shown a significant increase of o-α-syn in CSF of PD patients compared to controls(15-19), with an even better discrimination using the o-α-syn/t-α-syn-ratio(15, 16, 19). In blood, El-Agnaf et al(20) found an increase in plasma o-α-syn in PD patients, whereas

Park et al(17) did not find any increase in drug naïve patients. Wang et al(21) found an increase in the ratio of o-α-syn to the total amount of protein in red blood cells of PD patients. These results might indicate a superior diagnostic ability of o-α-syn analysis in bodily fluids compared to t-α-syn.

Using a fluorescence-based interaction assay, Reinke et al(22) identified 5 indole-based compounds that specifically targeted Aβ-oligomers, but not Aβ-fibrils. Using this approach, a fluorescence based interaction assay with a modified indole compound has been developed, targeting pre-fibrillar beta-sheet rich amyloid structures. Extracellular Aβ-plaques and intracellular neurofibrillary tangles of tau protein represent the pathological hallmark of AD(23). α-syn and tau proteins can interact promoting the polymerisation of each other, possibly promoted by Aβ-species(24). This creates a possible link between synucleinopathies and tauopathies.

In this study we applied this newly developed fluorescence assay to CSF and plasma from PD patients and controls. Our goal was to evaluate the usefulness of this fluorescence assay as a potential state biomarker, distinguishing a group of PD patients from controls. We will also evaluate its function as a rate biomarker, correlating the fluorescence score with clinical data from the PD group.

Method

Recruitment

26 PD patients were recruited from four different locations; the neurological departments in Sønderborg, Odense, and Roskilde as well as a private neurological practice. All patients apart from 1 were identified as PD patients by movement disorder specialists, with the one patient diagnosed as having Lewy body dementia (DLB). All PD patients met the United Kingdom Brain Bank Criteria for idiopathic PD(25).

15 controls were recruited from the neurological departments in Sønderborg and Odense. They underwent lumbar puncture as part of their diagnostic investigation (headache n=3, dementia n=3, multiple sclerosis n=3, vertigo n=1, ataxia n=1, trigeminal neuralgia n=1, sleep disorder=1, neuroborreliosis n=2).

Lumbar puncture

All lumbar punctures (LP) were performed on patients from 09.30-10.00 am after overnight fasting (21 of 26 patients). Patients on L-DOPA were instructed to (when possible) take their morning dose at 08.00 am, but time of intake varied from 05.30 am to 08.00 am. On patients the procedure was performed with the patient in a sitting position, using an atraumatic Pencan® (0,53x88mm) needle in the L3/L4 level, drawing out 13 ml CSF for routine analysis and storage for further analyses. On controls the procedure was performed from 09.30 am-12.15 pm using an atraumatic needle, and due to practical reasons it was not possible to ask controls to be fasting before the procedure. 6 of 16 patients had the procedure done in a sitting position without measurement of the CSF pressure, 10 had the procedure done in supine position with CSF pressure measurement before drawing CSF as part of their investigation. CSF was kept in a 15 ml polypropylene tube in ice water. Within 30 minutes the CSF was centrifuged at 2000g for 10 minutes at 4°C; it was then aliquoted into Saarstedt-tubes and put immediately on dry ice until storage at -80°C.

Plasma sampling

Blood was drawn after LP in the cubital fossa using a BD Vacutainer® Eclipse™ blood collection needle with pre-attached holder. 4 x 4,5 ml BD Vacutainer® tubes with citrate were filled, gently turned 4 times to mix blood with citrate and kept in room temperature (RT) until centrifuging. Tubes were centrifuged at 1800g for 10 minutes at RT. Plasma from the four citrate tubes were then aliquoted evenly into two 15 ml polypropylene tubes and centrifuged at 3000g for 10 minutes at RT. It was the aliquoted into Saarsted-tubes and put immediately on dry ice until storage at -80°C.

Clinical rating

Rating was performed on the same day as the LP procedure.

PD motor disease severity was rated in the ON-stage using the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part III(26), for patients taking L-DOPA it was performed approximately 50 minutes after intake. The procedure was video recorded for later assessment (apart from evaluating rigidity).

The Unified Dyskinesia Rating Scale (UDysRS)(27) objective score procedure was performed with video recording for later rating. For patients taking L-DOPA it was performed also approximately 50 minutes after L-DOPA-intake.

Cognitive rating was done using both the Mini-Mental State Examination (MMSE) and MOntreal Cognitive Assessment (MOCA).

Group division

Patients were divided into three groups. Patients not receiving L-DOPA but monoamine oxidase inhibitors or DA agonists (PD-N). Patients receiving L-DOPA but without LID (PD-L), patients receiving L-DOPA with LID (PD-LID), either by having a positive UDysRS objective score or a description of symptoms assessed as being L-DOPA-induced dyskinesia.

Routine CSF analysis

Routine analysis including glucose, protein and erythrocyte count was performed by the hospital departments of clinical biochemistry in Sønderborg, Esbjerg, Odense and Roskilde.

L-Tryptophanol (TROL) fluorescence assay:

The method was originally described in the PhD thesis by Glynn Jones (PhD, Aberdeen University)(28): The required mass of dry anhydrous Trol granules (Sigma) were dissolved in 50 ml 'glycine buffer' (50 mM glycine, pH 8 in 50 ml dH_2O) to make a 1 mM Trol stock. To increase the solubility of Trol samples were heated to 35°C whilst being stirred until granules dissolved, at which point pH was checked and adjusted to pH 8 if required. Following dissolution the Trol stock was 0.22 µm-filtered using polyethersulfone membrane sterile syringe fitted filters. The stock was further diluted using 0.22 µm-filtered glycine buffer and run at a final test concentration of 100 µM in all experiments.

Trol assays were carried out in UV colourless, UV star®, µclear® 96 well plates (Greiner, Germany) with signal level monitored over time (ΔT= 1 hour, Tmax= 3 hours). Samples were read from below ('bottom read') using a consistent gain of (900) and 10 flashes per well, using 280±10 nm and 360±10 nm, excitation and emission filters respectively.

BMG FLUOstar Omega is a band pass filter based system, as such rather than producing an excitation beam at a narrow range of wavelegths, it utilises band pass filters which allow a wider range of wavelengths to pass.

CSF and serum were assessed over 3 hour time courses, however due to stability of results T=1 hour was consistently used as the Trol results for each sample.

Statistical analysis

Statistical calculations were performed using Stata/IC 14.2 for Mac.
Comparisons between two groups were done using student's unpaired t-test for parametric numerical variables, Wilcoxon's rank-sum-test for non-parametrical numerical variables.
Comparisons between more than two groups were done using Kruskal-Wallis equality-of-populations rank test for non-parametric numerical values followed by Dunn's Pairwise Comparison for multiple comparison with Bonferroni adjustment. For parametric numerical data oneway ANOVA and subsequent Bonferroni multiple comparisons was used.
Multiple logistic regression was used to assess multiple correlations.
To evaluate the diagnostic ability of a test, receiver operating characteristics (ROC) was performed. Youden's index was calculated to find the optimal cutoff.
All statistical analysis was performed with and without DLB and AD cases.

Results:

Table 1:

	Control	PD-N	PD-L	PD-LID	PD total
Number of cases (n)	15	8	8	10	26
Age (years)	54.9 ± 11.3[a]	62,6 ± 6.6	67.4 ± 11.8[b]	59.9 ± 7.9	63 ± 9.1
Sex (F/M)	8/7	3/5	0/8	3/7	6/20
CSF protein (g/L)	0.34 ± 0.09[c]	0 .5 ± 0 .18	0.44 ± 0.14	0.45 ± 0.15	0.46 ± 0.15
UPDRS III		18.8 ± 6.8[d]	36.6 ± 7.5	30.1 ± 11.8	28.6 ± 11.5
UDysRS objective score		0	0	7.8 ± 5.7	3.0 ± 5.2
Symptom duration (years)		3.9 ± 2.4[e]	6.6 ± 3.7	8.8 ± 4.2	6.6 ± 4
Levodopa equivalent dose (mg)	10 ± 40[f]	293 ± 126[g]	663 ± 416	857 ± 288	624 ± 374
MOCA		28 ± 2.1[h]	25.7 ± 2.5	26.2 ± 1.6	26.7 ± 2.2
MMSE		29.2 ± 1.4	28 ± 3.7)	29.1 ± 0.7	28.8 ± 2.2

PDN; Parkinson's disease not receiving L-DOPA, PDL; Non-dyskinetic Parkinson's disease receiving L-DOPA, PDLID; Dyskinetic Parkinson's disease receiving L-DOPA, L-DOPA; Levodopa, F; female, M; male, UPDRS; Unified Parkinson's disease rating scale, UDysRS; Unified dyskinesia rating scale, MOCA; Montreal Cognitive Assessment, MMSE; Mini mental state examination, DLB; Lewy body dementia. Numerical values are described as mean ± SD.

[a] p=0.0042 vs PD-L

[b] p=0.0354 vs PD-LID when including DLB

[c] p=0.0055 vs PD total, p=0.0042 vs PD-N, p=0.0392 vs PD-L, p=0.0392 vs PD-LID

[d] p=0.0009 vs PD-L, p=0.0169 vs PD-LID

[e] p=0.0054 vs PD-LID

[f] One control received Ropinirol for restless legs

[g] p=0.0089 vs PD-L, p=0.0000 vs PD-LID

[h] p=0.0241 vs PD-L, p=0.0312 vs PD-LID

Demographic analysis:

Table 1 shows demographic data for controls and PD groups. Controls are only significantly younger than PD-L. Gender composition in the control group reflects the normal population, whereas the PD group shows a preponderance of men. Controls have lower CSF protein levels than all PD patients and PD-N distinctly.

In the PD group, PD-N has a lower UPDRS III score than PD-L and PD-L+PD-LID, as well as a lower LED than PD-L and PD-LID. A higher UPDRS III score (higher disability) is significantly correlated to the daily L-DOPA-equivalent dose (LED)(p=0.015). Non-L-DOPA-treated patients have higher MOCA scores than L-DOPA treated patients. Disease duration is significantly shorter in PD-N compared to PD-LID. LED is highly correlated to disease duration (p=0.004).

TROL scores in controls compared to all PD patients:

In Figure 1, Trol fluorescence for CSF and plasma and the plasma/CSF ratio are shown. CSF Trol scores are significantly lower in PD CSF (p=0.0203)(fig.1A). When excluding both the DLB and AD patients, the difference is not significant (p=0.0623), but only excluding the DLB patient, results are still significant (p=0.0289). Age is only significantly correlated with CSF Trol scores in the PD group when excluding the DLB patient (p=0.029), but not in controls. Neither gender, LED, MOCA scores, MMSE scores, nor UPDRS part III scores significantly correlated with CSF Trol scores.

Plasma Trol scores are significantly higher in PD compared to controls (p=0.0137)(Fig.2B). In controls plasma Trol scores are not significantly correlated to age or gender, but when including PD patients, age correlates with plasma Trol scores (p=0.003), and females have significantly lower plasma Trol scores (p=0.016). In a total multiple linear regression model using age and the binary values case/control and male/female, only age was significantly correlated to plasma Trol scores (p=0.04). Neither disease duration, LED, MOCA scores, MMSE scores, nor UPDRS part III scores correlated with plasma Trol scores.

The plasma Trol/CSF Trol ratio was significantly higher in PD compared to controls (p=0.0022)(Fig.1C). When looking at both controls and PD, females had significantly lower plasma Trol/CSF Trol ratios (p=0.005). Taking into account gender in a multiple linear regression analysis, PD patients still had significantly higher ratios (p=0.003).

Neither disease duration, LED, MOCA scores, MMSE scores, nor UPDRS part III scores correlated with plasma Trol/CSF Troll ratios.

TROL scores in controls compared to individual PD groups:

In Figure 2 TROL fluorescence data for CSF (Fig.2A) and plasma (Fig.2B) and the plasma/CSF ratio (Fig.2C) are shown for controls and the PD subgroups, PD-N, PD-L and PD-LID.

CSF TROL scores were significantly *decreased* only in PD-LID as compared to controls (p=0.006)(Fig.2A). Focusing on L-DOPA-treated PD as a single group, they have significantly lower CSF TROL scores than controls (p=0.003). Comparing dyskinetic to non-dyskinetic PD patients showed significantly lower CSF TROL in dyskinetic patients (p=0.0369).

Plasma TROL scores tended to be increased in PD-N and PD-L as compared to controls, but did not reach significance (Fig.2B). Neither dyskinesia status nor L-DOPA treatment significantly affected the plasma TROL score between PD patients.

Plasma Trol/CSF Trol ratios were significantly higher in PD-LID compared to controls (p=0.006).

CSF Trol/CSF total protein ratios

In Figure 3 CSF Trol fluorescence data are expressed as ratios over CSF total protein levels. The CSF Trol/CSF total protein ratios were significantly lower for the total PD group (p=0.0001)(Fig.3A), and also for all PD subgroups as compared to controls (PD-N: p=0.019, PD-L: p=0.031, PD-LID: p=0.007)(Fig.3B). Including both patients and controls, females had significantly higher CSF Trol/CSF total protein ratios than men (p=0.023), but taking gender into account in multiple linear regression analysis showed that disease status (case/control) was the only significant independent variable (p=0.000). Neither disease duration, LED, MOCA scores, MMSE scores, nor UPDRS part III scores correlated with this ratio.

In figure 4 an ROC analysis of CSF Trol/total protein ratios is shown. When excluding AD cases from controls and one DLB case from the total PD group, an optimal cut-off point at <2207 separates controls from PD with a 90% sensitivity and 72,73% specificity.

Neither in the control group or patient group was CSF Trol scores significantly correlated to the total protein concentrations in CSF.

Age effect:

In figure 5, age-dependent significant increases in plasma Trol in the PD group (Fig.5A) and for plasma in all subjects, including controls (Fig.5B).

Correlation between disease duration and CSF Trol scores.

Patients with longer disease duration have significantly lower CSF Trol scores (Fig.6). Taking dyskinesia status into account (PD-LID have significantly longer symptom duration and the lowest CSF Trol score) removes that significance. Multiple regression analysis including both disease duration and LED

Discussion:

The main findings of this study are:

(1) CSF Trol scores are significantly *lower* in PD compared to controls, whereas plasma Trol scores are *higher* in PD.

(2) The plasma/CSF Trol ratio and the CSF Trol/total protein ratio in CSF improves differentiation of PD from controls,

(3) Plasma TROL scores in PD is correlated to age and disease duration.

1.Decreased CSF TROL in PD:

One might expect that in PD oligomerization of α-syn and simultaneous aggregation of tau, Aβ and other amyloids would increase levels of oligomeric proteins in CSF. However, our data, indicating lower Trol scores in CSF of PD patients, suggest that lower levels of oligomeric proteins in the CNS may be released. Aggregation of defective proteins in PD may be the result of a dysfunctional autosomal-lysosomal pathway and as a result reduce its capacity to release oligomeric proteins into the CSF. The oligomerization of proteins such as Aβ and α-syn are concentration dependent(29, 30), and decreased CSF α-syn in PD as found in several studies(31-37), could thus be due to aggregation of the protein decreasing the amount of free α-syn. An animal study using radiolabeled Aβ-monomers and oligomers has shown that monomers are more rapidly cleared from the brain, and that clearance of Aβ-oligomers to CSF is markedly decreased(38). Also, a higher CSF oligomeric protein level in controls might reflect a generally lower intracellular concentration of oligomeric proteins preventing further aggregation, and a more efficient clearance of oligomeric proteins. In the glymphatic system (39), CSF flows in the subarachnoid space following an arterial paravascular route through the brain parenchyma. Through aquaporine 4 water channels in astrocytes there is an influx of water from the paravascular system into the interstitial fluid (ISF). A bulk flow drives the fluid through the brain parenchyma clearing solutes such as Aβ into a paravenous pathway. From there it can follow large calibre veins to cervical lymph nodes or again enter the CSF. Animal studies estimate that 11-30% of CSF is derived from bulk flow of ISF(40, 41). A decreased bulk flow through the ISF might decrease the amount of oligomeric proteins being driven towards the CSF, both explaining the lower CSF concentration of oligomeric proteins and potentially worsening of the on-going aggregation.

Specifically L-DOPA-treated PD patients had a significant decrease in CSF TROL. Could L-DOPA in itself induce aggregation of susceptible proteins? Potentially due to decreased activity of the enzyme aldehyde dehydrogenase as well a decreased vesicular uptake of dopamine(42), an increase of the naturally occurring dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) has been found in brain samples of PD patients(43). DOPAL is highly neurotoxic, especially to dopaminergic neurons(44), and increases the aggregation of α-syn into oligomeric species(45). Using high performance liquid chromatography identifying dopamine and metabolites in the same PD group (46) we found the PD-LID to have a significantly higher level of dopamine compared to the metabolite DOPAC, which is the metabolite of DOPAL. An increased production of DOPAL in this group might explain why they have the lowest CSF TROL score of all patients. Or possibly L-DOPA metabolites interfere with the Trol-assay directly.
It has to be noted that this assay is unspecific as to what species of prefibrillary proteins are measured. The TROL fluorescence in controls may represent a different composition of proteins than in PD.

2. Increased plasma TROL in PD:

Oligomeric proteins are ubiquitous and play many roles in the normal functioning of e.g. cellular membranes. One of the motifs of protein oligomerization is the very stable β-sheet structure, which has the risk of polymerizing into an amyloid formation(47). Our findings do not indicate what type of oligomeric protein might be increased in patients.
This could indicate a peripheral source for production of oligomeric proteins in PD:
In PD, a systemic affection of peripheral nervous system (PNS) has been proposed. Several studies have focused on peripheral α-syn aggregation in PD, identifying phosphorylated α-syn in submandibular glands(48), α-syn in salivary glands(49), intraneuronal phosphorylated α-syn in skin biopsies (50) and intraneuronal α-syn aggregation in the enteric nervous system (ENS) of the gastric mucosa (51, 52). Affected neurons in the PNS (e.g. ENS) might release a larger amount of oligomeric proteins into the bloodstream due to the continuous aggregation of the ubiquitous α-syn and a more direct connection to the blood stream. It could be speculated that other amyloid species apart from α-syn are simultaneously aggregated into a prefibrillary formation and released into the blood stream.

3. Decreased oligomeric/ total protein ratio in CSF of PD

Neither patients nor controls had visual blood mixture in CSF during lumbar puncture, and none had a marked increase of CSF erythrocytes (results not shown). Albeit still in the normal range, significantly higher protein levels were measured in the CSF of PD patients. This may be due to a) a more permeable BBB allowing influx of proteins from the blood stream or b) neuroinflammation, which increases CSF protein levels. One study did show an increased CSF/serum albumin and CSF/serum IgG ratio in advanced phases of PD(53), indicating a potential breach of the BBB.

Thus, a possible explanation for the decreased oligomeric/total protein ration in CSF of PD patients is the combination of a disrupted BBB, neuroinflammation and a dysfunctional clearance of oligomeric proteins from brain cells. Do oligomeric proteins from the blood stream would cross the BBB as well? If so, a possible explanation for our findings could be that a decreased bulk flow of the ISF would allow for peripheral oligomeric proteins crossing the BBB to aggregate in the brain parenchyma before reaching the CSF, thus explaining the differences in ratio.

4. Disease duration and age correlations:

Patients with the longest disease duration (PD-LID) have significantly lower CSF TROL scores and CSF TROL is negatively correlated to disease duration, which means that CSF TROL scores might reflect the gradual increase of protein aggregation, thus potentially functioning as a rate biomarker, decreasing over time. Due to the potentially augmenting effect of L-DOPA on protein aggregation mentioned earlier, daily L-DOPA intake might be a major confounder. Increased disease duration means further neurodegeneration necessitating larger doses of L-DOPA, and LED is highly correlated to disease duration, even though a direct correlation between LED and CSF TROL is not found.

CSF TROL was not correlated to severity of motor symptoms (UPDRS III); this could be due to the significant effect of L-DOPA treatment on motor symptoms. If possible, future studies should include CSF samples from patients after L-DOPA washout, rating severity of motor symptoms in the OFF-stage.

Plasma TROL scores are significantly correlated to age, even when including controls, but not in controls by themselves. It seems plausible that the amount of oligomeric proteins in plasma increases due to the normal ageing process. Supporting this hypothesis, Foulds et al (2013)

found a significant increase of total α-syn in plasma over time in PD patients(54). Even then, taking age into account, PD-N still had significantly higher scores than controls.

Comments to results:

The study includes only a small number of PD patients. The controls were not completely age matched for the PD-N and PD-L groups, and a subgroup of controls had not been fasting and had the spinal tap performed in a lying position; this might affect measurements. Also the gender distribution is skewed, although we didn't find any gender effect on either CSF or plasma TROL.

In terms of correct PD diagnosis, movement disorder specialists have diagnosed and done regular follow-ups on all the clinically well-defined patients in this study.

Conclusion:

In this study, for the first time to our knowledge, the indole-based compound L-Tryptophanol has been used to quantify prefibrillary proteins in CSF and plasma of patients with a neurodegenerative disease.

PD patients have lower Trol scores in CSF and higher Trol scores in plasma. The ratio between CSF Trol and total CSF protein concentrations shows a promising ability to distinguish between PD and controls.

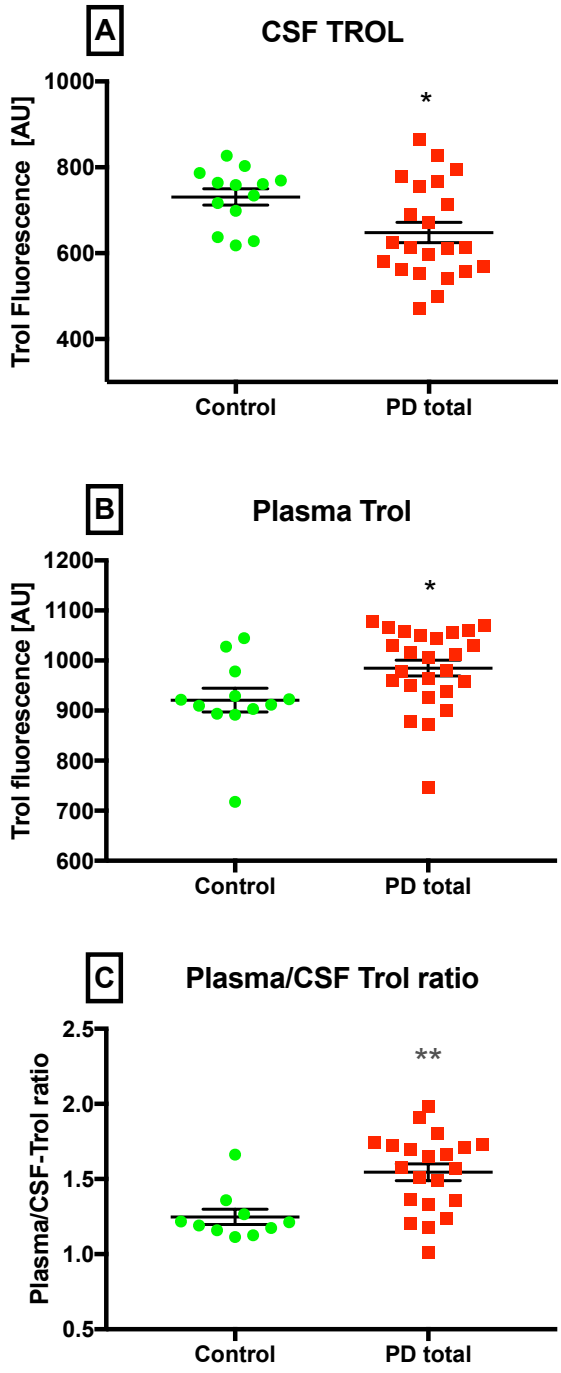
Especially non-L-DOPA treated PD patients have significantly higher plasma concentrations of oligomeric proteins than controls.

The CSF concentration of oligomeric proteins decreases as the disease progresses.

Thus, the combination of CSF and plasma measurements of the total oligomeric protein concentration shows potential as both a rate and state biomarker.

Further studies are needed to identify which protein species are detected by the assay and whether other constituents (e.g. medicine or metabolites) interfere with the Trol assay. We suggest future studies using this assay, including a larger group of PD patients with a wide disease spectrum. Follow up studies would corroborate whether the CSF TROL score can be used as a marker for disease progression, although the potential effect of L-DOPA would have to be taken into account. Including a larger group of PD patients with early symptoms would assess the function of this assay as an early biomarker for PD.

Figure 1: CSF TROL, plasma TROL scores and plasma/CSF TROL ratios in controls and PD:

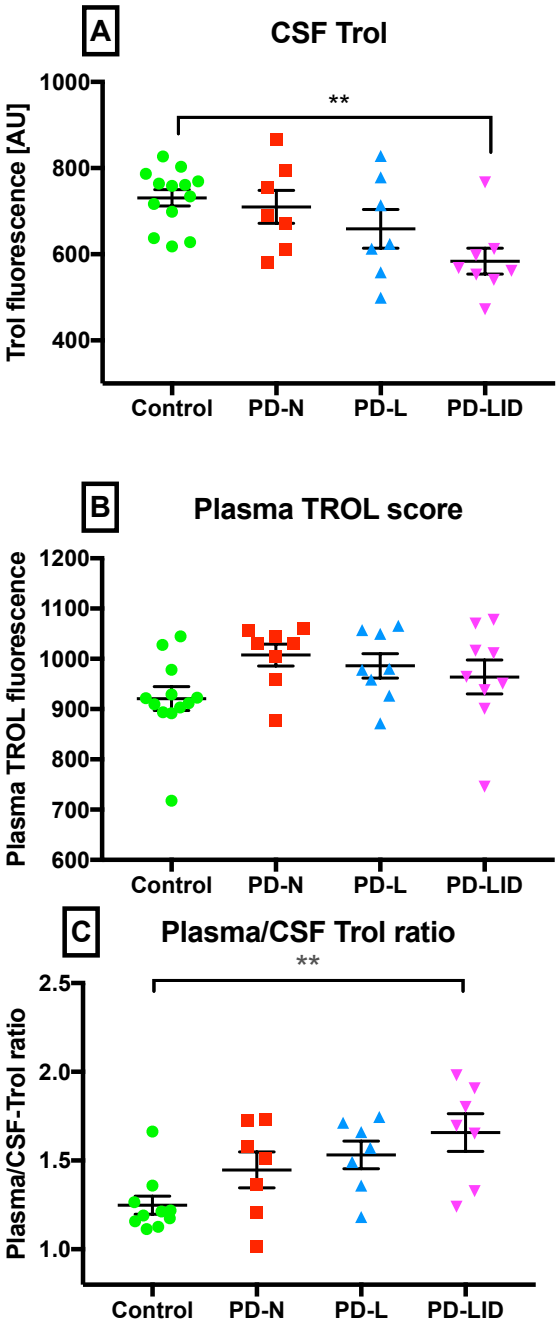


PD; Parkinson's disease, Trol; L-Tryptophanol

(A) Student's t-test, p=0.0203 (B) Wilcoxon ranksum test, p=0.0137 (C) Student's t-test, p=0.0022; Data presented as mean ± standard error of mean.

* $p<0.05$ ** $p<0.01$

Figure 2: Differences in CSF and plasma TROL scores and CSF/plasma TROL score ratio between controls, PD-N, PD-L and PD-LID.

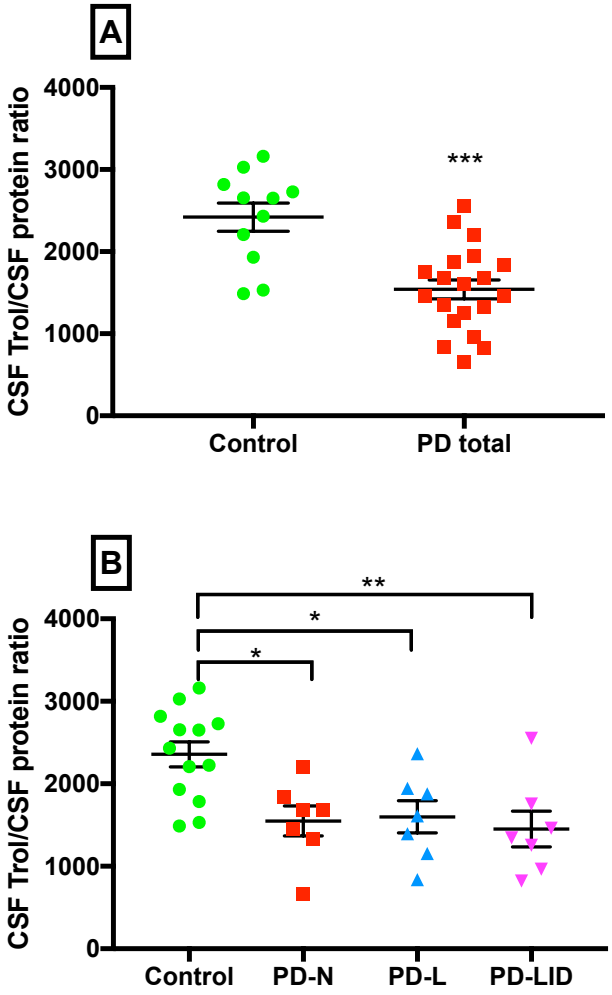


PD-N; Parkinson's disease not receiving L-DOPA, PD-L; Parkinson's disease receiving L-DOPA, non-dyskinetic, PD-LID; Parkinson's disease receiving L-DOPA, dyskinetic.

* $p<0.05$ ** $p<0.01$

(A) ANOVA p=0.0120, subsequent Bonferroni multiple comparison (B) ANOVA not significant, subsequent Dunn's test with Bonferroni correction for multiple comparisons (C) ANOVA p=0.0155, subsequent Bonferroni multiple comparison.

Figure 3: Ratio between CSF Trol score and CSF total protein concentration in controls compared to PD and compared to individual PD groups.

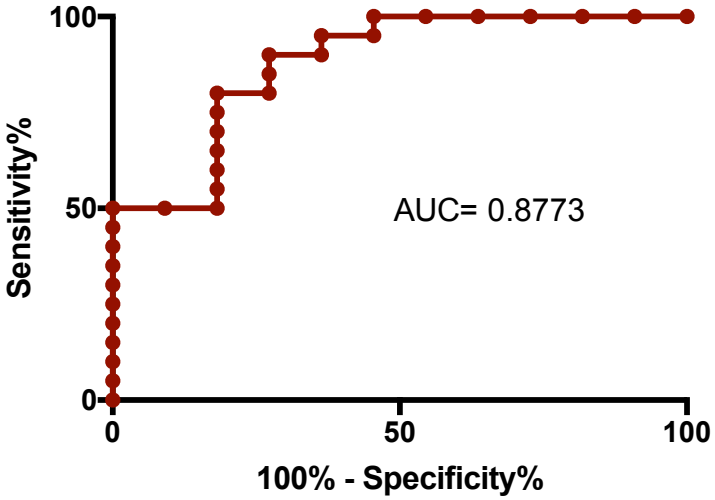


PD-N; Parkinson's disease not receiving L-DOPA, PD-L; Parkinson's disease receiving L-DOPA, non-dyskinetic, PD-LID; Parkinson's disease receiving L-DOPA, dyskinetic.

* p<0.05 ** p<0.01 *** p<0.001

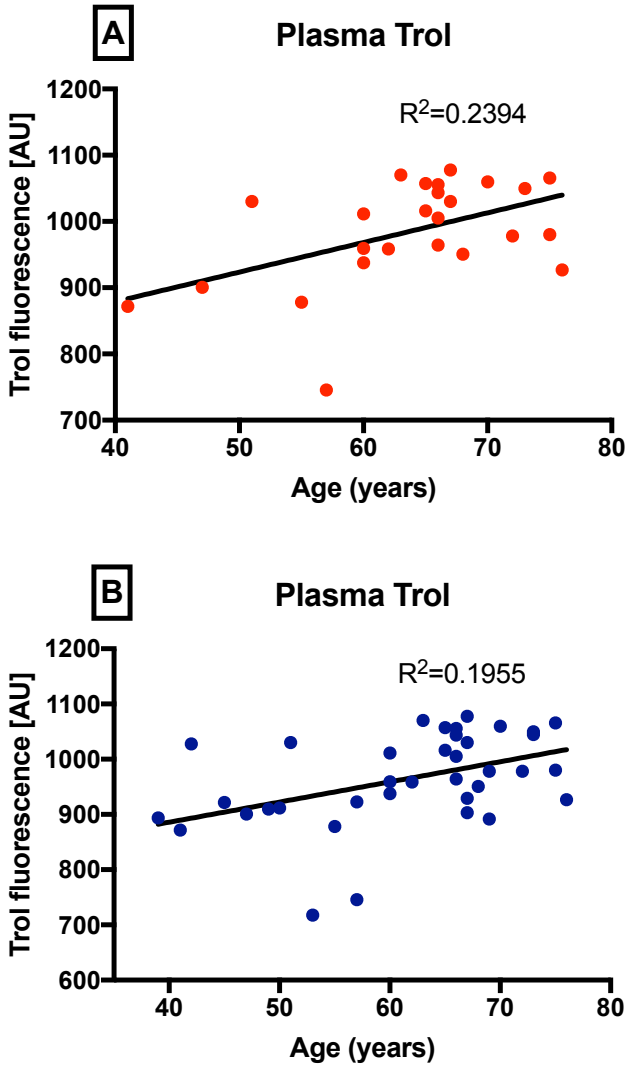
(A) Student's t-test (B) ANOVA p=0.0017, subsequent Bonferroni multiple comparison

Figure 4: ROC analysis of plasma/CSF TROL score ratio differing PD from controls



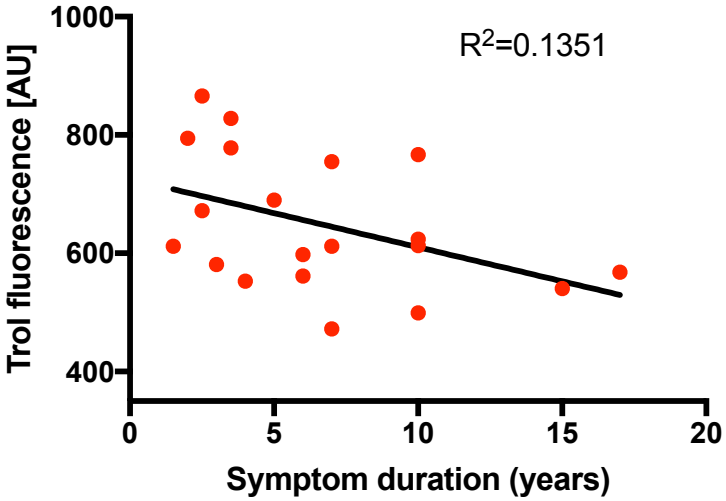
p=0.0006; ROC calculated excluding AD and DLB cases. At the optimal cutoff point at <2270 the CSF Trol/CSF protein ratio has a sensitivity of 90% and specificity of 72.73

Figure 5: Correlation between age and plasma Trol scores in PD without (A) and with (B) controls.



(A) and (B) shows the correlation between age and plasma Trol respectively with and without controls ((A) p=0.012 (B) p=0.003). Linear regression analysis with bootstrapping.

Figure 6: Correlation between disease duration and CSF Trol scores:



The figure shows the significant correlation (p=0.027) between disease duration and CSF Trol scores. Linear regression analysis with bootstrapping.

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APPENDIX V-IX

Appendix V

International Parkinson and
Movement Disorder Society

States that

Andreas Dammann Andersen

has passed the

MDS-UPDRS Training Program & Certificate Exercise

on 19 August 2015

Christopher G. Goetz, MD
Chair, MDS-UPDRS Training Program
& Certificate Exercise
Rush University Medical Center

Oscar S. Gershanik, MD
President
International Parkinson and Movement
Disorder Society

Exam ID: 7360

Appendix VI

The *Movement* Disorder Society

Certifies that

Andreas Dammann Andersen

has passed the

MDS-UDysRS Training Program & Certificate Exercise

on 31 August 2015

Christopher G. Goetz, MD
Chair, MDS-UDysRS Training Program
& Certificate Exercise
Rush University Medical Center
Chicago, IL

Günther Deuschl, MD
President
The *Movement* Disorder Society

Advance.
Improve.
Educate.
Collaborate.

International Parkinson and Movement Disorder Society

December 22, 2016

Andreas Dammann Andersen
University of Southern Denmark
Institute for Regional Health Research
J.B. Winslows Vej 19, 3.
Odense, Denmark 5000
T: +4530226030
dammanden@gmail.com

Re: Letter of Agreement for Authorization to Use Materials Owned by the International Parkinson and Movement Disorder Society (MDS)

Dear Dr. Andersen:

Thank you for your request for authorization to use the MDS-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) and the Unified Dyskinesia Rating Scale (UDysRS) in your PhD project entitled, "Cerebrospinal fluid biomarkers for Parkinson's disease and L-DOPA - Induced Dyskinesia", in collaboration with the Hospital of Southern Jutland and the University of Southern Denmark with Dr. Andreas Dammann Andersen serving as Project Manager. This project is identified by the protocol number S-20130098, and the Danish Data Protection Agency identification number 1224.

The International Parkinson and Movement Disorder Society (MDS) approves your request for this one-time use of the MDS-UPDRS in English and the UDysRS in English only contingent upon payment of the government/non-profit rate of $1,000 USD per language, per scale for a cumulative total of $2,000 USD, and the additional provision that the user recognizes this scale as a product of MDS and references the scale appropriately.

By submitting your request, you agree to the following terms:

○ I understand that the MDS-UPDRS and UDysRS may only be used for the purposes described above. I also understand that reproduction, translation, modification, sale, or distribution of any portion of the MDS Rating Scales is strictly prohibited and, specifically, that the MDS Rating Scales may not be incorporated into clinical trials, training or certification programs or materials, software programs, or otherwise except through use of the Permissions Request form and payment of applicable fees. Further, MDS shall have no liability related to use of any Rating Scale, and I hereby release, hold harmless, and indemnify MDS, its officers, directors, employees, volunteers, and agents, from any loss, damage, or claim based on such use.

○ I understand that MDS does not grant permission to translate the MDS-UPDRS or UDysRS. Further, inclusion, or publication of data obtained from the MDS-UPDRS or UDysRS that has been independently translated is strictly prohibited.

Please confirm your agreement with these terms by signing below and returning a copy of the signed agreement with your payment and a copy of the invoice to the MDS International Secretariat at:

International Parkinson and Movement Disorder Society
Attn: Jennie Socha, Executive Director
555 E. Wells Street, Suite 1100
Milwaukee, Wisconsin 53202 USA
Fax: +1 414-276-3349

Please do not hesitate to contact me with any questions or concerns.

Advance.
Improve.
Educate.
Collaborate.

Sincerely,
Jennie Socha
Executive Director
International Parkinson and Movement Disorder Society

Agreed to and accepted by:

INTERNATIONAL PARKINSON AND MOVEMENT DISORDER SOCIETY, INC.	**UNIVERSITY OF SOUTHERN DENMARK**
	
(Signature)	(Signature)
Name (Print): Jennie Socha	Name (print):
Title: Executive Director	Title:
Organization: International Parkinson and Movement Disorder Society	Organization:
Date: December 22, 2016	Date:

International Parkinson and
Movement Disorder Society

Measuring α-synuclein-content in erythrocytes (RBC) november 2016 at Statens Serum-Institut (SSI)

Sample preparation

RBC thawed at room temperature (RT) and carefully mixed.
5 µL samle mixed with 95 µL diluted PBS (1 part PBS 10X + 99 parts MilliQ)
Put on cold storage over night for hemolysation.
Then diluted in PBS (1 part PBS 10X + 9 parts MilliQ) for the dilution 1:1000.

ELISA Assay

MaxiSorpplade coated with 100 µL 5 µg/mL HYB 366-03 per well.
Put on cold storage over night.
Washed 3x 60 seconds with washing buffer on shake table at 200-300 rpm.

Standard row placed in duplicate 100 µL/well: Recombinant α-synuclein diluted in PBS to the following concentrations: 0.00, 0.020, 0.040, 0.060, 0.080, 0.10, 0.12, 0.14 µg/mL
Samles placed in duplicate 100 µL/well.
Incubated for 60 minutes on shake table at 200-300 rpm.
Washed 3x 60 seconds with washing buffer on shake table at 200-300 rpm.

100 µL/well biotinylated HYB 366-01 diluted 1:200 in washing buffer.
Incubated for 60 minutes on shaking table at 200-300 rpm.
Washed 3x 60 seconds with washing buffer on shaking table at 200-300 rpm.

100 µL/well streptavidin-AP diluted 1:1000 in washing buffer.
Incubated for 60 minutes on shaking table at 200-300 rpm.
Washed 3x 60 seconds with washing buffer on shaking table at 200-300 rpm.

100 µL/well pNPP diluted in substrate buffer for AP to 1 mg/mL.
Incubated for 30 minutes on shaking table at 200-300 rpm.
Measured at 405 nm / 620 nm on ELISAreader from TECAN.

PBS 10X delivered by SSI. Ref: 24234.
Carbonate buffer pH 9,6 delivered by SSI. Ref: 29779.
Washing buffer pH 7,2: PBS with 0,1% Triton-X100.
Substrate buffer for AP pH 9,8 delivered by SSI. Ref: 63167.
HYB 366-03: Monoclonal mouse anti-α-synuclein.
Recombinant α-synuclein from Alpha Diagnostics International. Ref: SYN11-R.
HYB 366-01: Monoclonal mouse anti-α-synuclein.
Streptavidin-AP from DakoCytomation. Ref: D0396.
pNPP from Sigma Life Science. Ref: 1001964484.

APPENDIX IX

METHOD FOR GLUCOCEREBROSIDASE (GCase) FLUORESCENCE ASSAY

Results used from the GBA assay are based on two separate runs. Differences between the two runs will be noted in the following description.
CSF samples were thawed on ice. MUGIc (4-Methylumbelliferyl beta-D-glucopyranoside)(Sigma, M3633) was used as the GBA substrate.

Reaction buffer (for two reading plates):
10 ml of citrate/phosphate reaction buffer was made by mixing 0.2 g 0.2% taurodeoxicholate (Sigma, T0875), 2.94 g sodium citrate (Merck, 1.06448.1000), sodium phosphate (Merck, 1.06346.1000) and 90 ml dH_2O. 16.93 mg of MUGIc was added in the dark to prevent breakdown. pH was adjusted to 5.2 using NaOH. In run two the reaction buffer was alcalic and pH was adjusted to 5.2 using 3 ml 1M citric acid. The mixture was filled to 100 ml using dH_2O. 100 microliter triton-x-100 was added to the mixture. The mixture was put on a whirlmixer until use within 30 minutes.

Stock of standard:
4-Methylumbelliferone (Sigma, M1381) was used as standard. 1 mg 4-Methylumbelliferone was mixed with 1 ml Methanol and subsequently diluted in Ringers to a final concentration (µM) on the assay plate of 10, 5, 1.67, 0.56, 0.19, 0.06, 0.02 and 0 (100% Ringers).

Stop solution:
Prepared by mixing 15 g glycine (Sigma, G7126) and 8 g NaOH (Merck, 1.06498.1000) in 450 ml water. pH was adjusted to 10.8 with 10M NaOH in first run. In the second run pH was too high (pH 11.25) and was corrected with 3 ml 10M citric acid to pH 10.8.

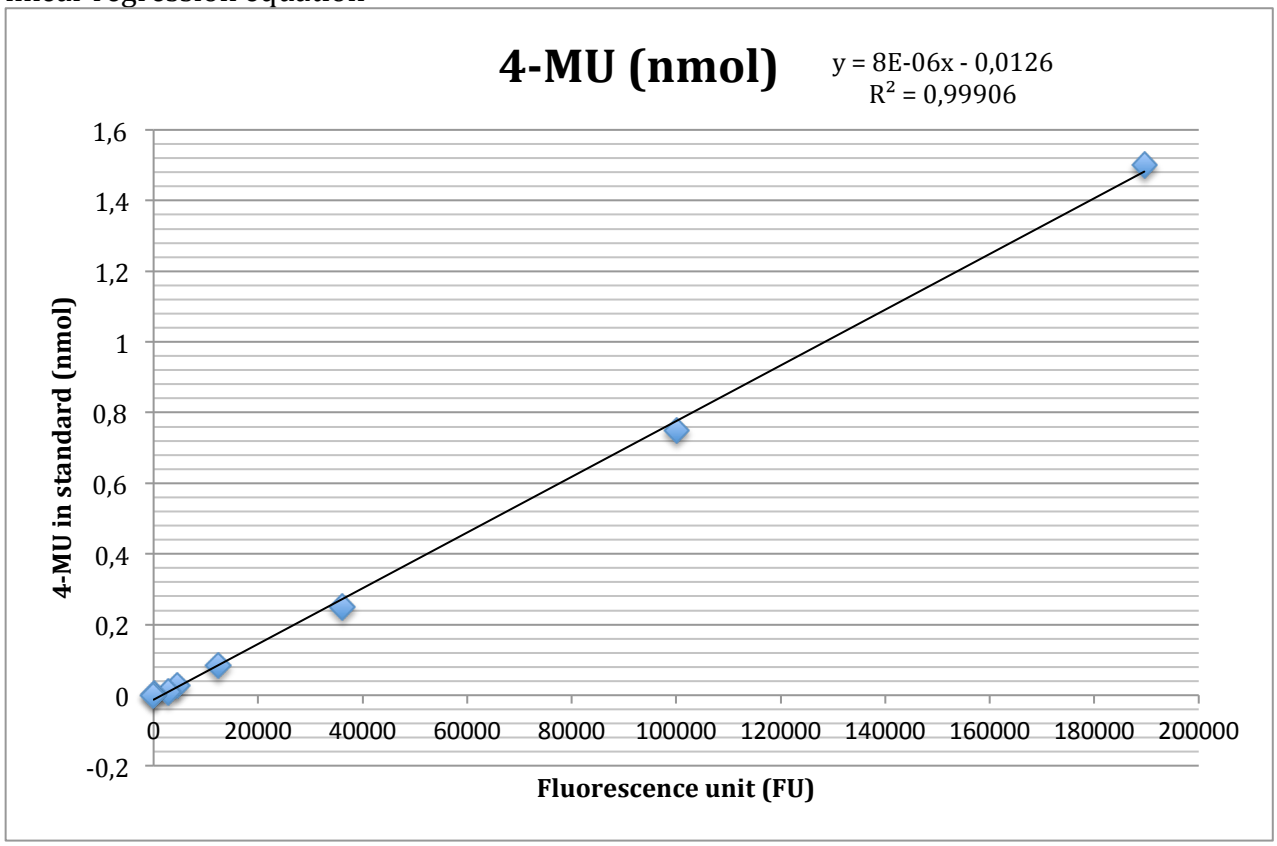
Plate preparation:
25 µL of each standard was added in triplicates to wells. 25 µL thawed CSF was added in triplicates. In all wells being used, 50µL of substrate (MUGIc/reaction buffer) was added. The plate was covered and incubated in the dark at 37°Celcius for 24 hours.

Plate reading:
For both runs the enzyme reaction was stopped after 24 hours by adding 75 µL of stop solution to each well being used.
In the first run the plates were read on an Enspire 2300 multimode plate reader (Perkin Elmers inc., Waltham, Massachusetts, USA) with software version 4.1 (excitation wavelength 365 nm, emission wavelength 445 nm).
In the second run the plates were read on a BMG Labtech Fluostar Omega (BMG Tech, Ortenberg, Germany) with built in software (same excitation and emission wavelengths) (with kind assistance from Ditte Neess Pedersen (MSc) and Lars Duelund (MSc), Institute of Biochemistry and Molecular Biology).

Calculated concentration:
The mean fluorescence unit (FU) of the triplicate standard concentrations was corrected (corrected fluorescence, CF) by subtracting the mean FU of the 0 concentration (100% Ringers) well. CF values are used to create a standard curve (CF as x-axis, and the amount of

standard (nmol 4-MU) added as the y-axis). Linear regression is used to identify the y-intercept (α) and slope (β) of the standard curve (see fig.1).

Figure 1: Standard curve for calculating actual amount of 4-MU in samples including simple linear regression equation



4-MU; 4-methylumbelliferyl

The corrected sample fluorescence value (CSF) is calculated by subtracting the mean blank sample background FU from the mean sample FU. To calculate the amount (nmol product) in samples after 24 hours, CSF values are plotted in the standard curve:

nmol 4-MU = CSF * β + α.

Enzyme activity was then determined in units (U), with one U being defined as the amount of enzyme that hydrolyzes 1 nmol of substrate pr. minute at 37°C.
Values were converted to mU/mL by the following equation:

GCase activity (mU/mL) = (nmol 4-MU)/(24*60)*1000/0.025

To compare the two runs that were analysed on different platereaders, we normalized the data by dividing the actual GCase activity with the control mean GCase activity:

normalised GCase activity = actual GCase activity/ GCase activity (control mean)